Molecular Characterization and Functional Analysis of an AGAMOUS-like Gene CiAG from Pecan

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Abstract. The floral homeotic C-function gene AGAMOUS (AG) has been shown to be critical in the determination of stamen and carpel identity in Arabidopsis. In the present study, a new homologue of AGAMOUS gene from pecan [Carya illinoinensis (Wangenm.) K. Koch], denoted by CiAG, was isolated and its function was characterized. The complementary DNA (cDNA) of CiAG contains an open reading frame of 687 base pairs (bp) encoding 227 amino acids. Multiple sequence comparisons revealed that CiAG had the typical MIKC structure. Phylogenetic analysis indicated that CiAG is closely related to C-lineage AG. The expression of CiAG was highly accumulated in the reproductive tissues (staminate flowers, pistillate flowers, and fruitlets) than in vegetative tissues (leaves and current-growth branches). Arabidopsis overexpressing CiAG exhibited earlier flowering. The homeotic transformations of petals into stamen organs were observed in 35S::CiAG transgenic plants. All these results indicated that CiAG plays a key role in the process of flower development of pecan.

A number of MADS-box genes are involved in the control of the development and specification of flower organs in higher plants. In the well-known ABC model (Coen and Meyerowitz, 1991), three classes of genes, A, B, and C functions, specify the four organ types of the typical angiosperm flower. According to this model, sepal identity is specified by the A function alone, petal specification of flower organs in higher plants. In the third whorl, AG functions in the specification of stamens; in the fourth whorl, C-function MADS-box gene plays a key role both in stamen and carpel formation, we isolated a cDNA sequence of CiAG in this study. The expression profiles of CiAG gene in reproductive tissues (staminate flowers, pistillate flowers, and fruitlets) and vegetative tissues (leaves and current-growth branches) of three varieties (‘Shaoxing’, ‘Pawnee’, and ‘Mahan’), were investigated by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). We then transformed the CiAG gene into Arabidopsis to study the function of CiAG on flowering.

Materials and Methods

Plant materials. The grafting seedlings of pecan were grown in Nanjing, Jiangsu province, China. Staminate flowers, pistillate flowers, fruitlets, leaves, and current-growth branches were collected from the 6-year-old cultivars of Mahan, Shaoxing, and Pawnee in May. The tissues were collected from the same direction of each tree, three trees as three replicates. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C until used.

Cloning of CiAG. Total RNA was extracted from the staminate flowers of ‘Mahan’ using The Plant Total RNA Extraction Kit (BioTeke, Beijing, China). First-strand cDNA was synthesized from 1 μg total RNA with an oligo(dT)18 adapter primer using PrimeScript RTase (TaKaRa, Japan).
RT-PCR was performed using the length of the coding region sequences, and the PCR product was cloned and sequenced. To ensure the full length cDNA of the fragment 1 which belonged to AGAMOUS group (Mo et al., 2013). The gene-specific primers GSP1 (5′-CAGTAC TAATCGTAAGTCACCTTCTG-3′) and GSP2 (5′-CTTCTTGTAAGGGCCGA CGGCTT-3′) were designed based on the conserved MADS domain nucleotide sequence. Nested PCR was carried out to ensure the full length cDNA of the fragment 1 which belonged to AGAMOUS group (Mo et al., 2013). The gene-specific primer GSP1 and an abridged universal amplification primer AUAP (5′-CGGCTT-3′) were used for first-round PCR, and the gene-specific primer GSP2 (5′-GAGGCGCAACGGCTT-3′) were used for second-round PCR using the first-round PCR product. The cycling program consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product was then cloned into the pMD19-T simple vector (TaKaRa, Japan) and sequenced. To ensure the full length of the coding region sequences, RT-PCR was performed using the VvAG-F (5′-ATGGGGAGGGGGAGGATAGAAA-3′) and VvAG-R (5′-CGCCATAACAGGGCA AGAATCTTCTGTAAGAGGCGCAA TCCTGTAA -3′) and the PCR product was cloned and sequenced.

Amino acid alignment and phylogenetic analysis. The deduced amino acid sequences were analyzed in the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) for searching the protein sequences of homologues. Alignment of deduced amino acid sequences was performed by using the Clustal W multiple alignment program of the BioEdit software. A phylogenetic tree was constructed using the neighbor-joining method in the MEGA 5.1 software with 1000 replication bootstrap tests. The accession numbers of the protein sequences used in this study are as follows: Juglans regia, JrAG (CAC38764); Corylus heterophylla, ChMADS1 (AEU08497); Prunus persica, PpMADS4 (AAU29513); Momordica charantia, McMADS2 (ABC25564); Citrus sinensis, CsAG (ADP02394); Prunus serotina, PsAG (ACH179274); Jatropha curcas, JcAG (AAE11211); Gossypium hirsutum, GhMADS5 (ABM69043); Petunia xhybrida, FBPI (CAAS7311) and FBPII (CAAS7445); Arabidopsis thaliana, AGAMOUS (NP_567569), AP1 (NP_177074), CAL (Q90801), AGL8/FUL (NP_568929), AGL79 (NP_189645), PI (NP_197524), AP3 (NP_191002), AGL11 (NP_192734), SEP1 (NP_568322), SEP2 (NP_186880), SEP3 (NP_850953), and SEP4 (NP_849930).

Gene expression analysis of CiAG in pecan using qRT-PCR. Total RNA isolation was done as described above. Reverse transcription was performed using 1 μg of total RNA with the ReverTra Ace qPCR RT Kit (TOYOBO, Code No. FSQ-101) according to the manufacturer’s instructions. The gene-specific primers for CiAG were QTAG-F: 5′-AGGGTCTCAGTCTGAGCACAAC-3′ and QTAG-R: 5′-GGTCTCCTCATTCCTCCG GTA3′. The Cq value was used as a positive internal control with primers ACTIN-F (5′-GCTGAACGGGAAAAATTGTC-3′), and ACTIN-R (5′-AGAGAATCGTGGAAAGAGGA-3′). qRT-PCR was carried out on the Applied Biosystems 7300 Real Time PCR System with a 20 μL reaction volume, containing 1 μL 10-fold diluted cDNA, 0.3 μL (10 pM) of each primer, 10 μL SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa Code: DRR041A), and 8.4 μL sterile double-distilled water. The PCR conditions consisted of denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 20 s, 57 °C for 20 s, and 72 °C for 40 s. The specificity of the individual PCR amplification was checked using a heat dissociation curve from 55 to 95 °C following

![Fig. 1. Sequence comparison of CiAG and the Ag-related MADS domain proteins. The MADS domain and the K domain are marked. The region between the MADS- and K-domain is I domain. The Ag motifs (AG I and II), which are highly conserved regions reported by Kramer et al. (2004), C-terminal regions are boxed. Arabidopsis thaliana, AGAMOUS (NP_567569); Corylus heterophylla, ChMADS (ABM69043); Prunus persica, PpMADS4 (AAU29513); Momordica charantia, McMADS2 (ABC25564); Citrus sinensis, CsAG (ADP02394); Prunus serotina, PsAG (ACH179274); Jatropha curcas, JcAG (AAE11211).](image-url)
the final cycle of the PCR. The relative levels of genes to control CiActin messenger RNAs were analyzed using the 7300 system software and the 2^ΔΔCT method (Livak and Schmittgen, 2001).

Construction of expression vector and Arabidopsis transformation. PCR amplified full-length cDNA of CiAG fragments, using primers which introduced a BamHI or Kpn I restriction site, were cloned into pMD19-T, and recombinants were verified by sequence analysis. The fragments were then ligated into the corresponding sites of the binary vector of pCAMBIA1301 under the control of Cauliflower mosaic virus (CaMV) 35S promoter in the sense orientation. The CiAG binary constructs were introduced into Agrobacterium tumefaciens strain EHA105 using a freeze-thaw method and verified with PCR. The 35S::CiAG constructs were transformed into Arabidopsis (Clough and Bent, 1998). The transgenic Arabidopsis lines were selected in the half-strength MS medium containing hygromycin (20 μg/ml). Survival transgenic plants were grown in the greenhouse at 22 °C under long-day (16-h photoperiod) exposure and further verified by RT-PCR analyses. The T2 and T3 generations were used for phenotypic assessment.

Results

Isolation and sequence analysis of CiAG. Using RT-PCR and RACE strategy, the full length cDNA of CiAG was obtained. The CiAG amino acid sequence was aligned with its homologues using the NCBI BLAST program, and the best hit was J. regia AGAMOUS, an AG-like gene. This suggested that this isolated cDNA clone represents an AG-like gene from C. illinoinensis, so we named this gene CiAG. The cDNA is 1013 bp, with an open reading frame of 684 bp that encodes a 227–amino acid protein which shares high homology with ChMADS from C. heterophylla (88% identities), PsAG from P. serotina (83% identity), and JcAG from J. curcas (83% identity). A multiple alignment of CiAG with other MADS proteins (Fig. 1) indicated that the CiAG contains a highly conserved MADS domain (2–57), a short I region (58–90), a half-conserved K domain (91–173), and the characteristic conserved C-terminal region (174–227). Moreover, CiAG has the AG motifs I and II in the C-terminal (Kramer et al., 2004). Phylogenetic analysis (Fig. 2) showed that CiAG is closely related to C-lineage AG homologues, while sharing low similarity with A, B, D, and E lineages.

CiAG expression pattern. qRT-PCR was performed to detect the expression pattern of CiAG in vegetative tissues (leaves and branches) and reproductive tissues (staminate flowers, pistillate flowers, and fruits) of the three cultivars (Mahan, Pawnee, and Shaoxing). The results indicated that the transcript of CiAG can be detected in both vegetative tissues and reproductive tissues (Fig. 3). In the three different cultivars, the expression levels were strong in reproductive tissues, and were barely detectable in vegetative tissues (Fig. 3).

Phenotypes of CiAG overexpression in Arabidopsis. Functional analysis of CiAG was investigated by its ectopic expression in Arabidopsis. The 35S::CiAG transgenic Arabidopsis was obtained through hygromycin screening, and ectopic expression of CiAG was confirmed by RT-PCR analysis from five independent transformation events (Fig. 4); the flowering time and phenotypic properties of T2 and homozygous T3 plants were studied. Five of 26 independent T2 lines were selected. In wild-type plants, the organs of the second whorl were homeotically changed, the bases of the petals were thin and transformed homoeotically into filament-like structures (Fig. 5H), whereas the sepal were curled and had no obvious homeotic changes.

**Fig. 2.** Phylogenetic analysis of AG-like MADS-box proteins. CiAG from pecan is boxed. Prunus persica, PpMADS4 (AAU29513); Prunus serotina, PsAG (ACT72947); Monordica charantia, McMADS2 (ABC25564); Jatropha curcas, JcAG (AAE11211); Corylus heterophylla, ChMADS1 (AEU08497); Carya illinoinensis, CiAG; Juglans regia, JrAG (CACP08746); Citrus sinensis, CsAG (ADP02394); Arabidopsis thaliana, AGAMOUS (NP_567569), AGL11 (NP_192734); Gossypium hirsutum, GhMADS5 (ABM69043); Petunia hybrida, FBP7 (AAA57311); FB11 (AAA57445); SEP4 (NP_849930); SEP3 (NP_850953); SEP1 (NP_568322); SEP2 (NP_186880); AG1 (NP_189645); AGL8/FLA (NP_568929); AP3 (NP_177074); CAL (Q9R081); PI (NP_197524); AP3 (NP_191002).
A new MADS-box gene, CiAG, was isolated from pecan, in this study. Deduced amino acid alignment and phylogenetic analysis revealed that CiAG has the typical M, I, K, and C domain and is closer to the AGAMOUS-like gene from walnut (Figs. 1 and 2). However, CiAG lacks the N-terminal domain preceding the MADS-box. The initiator methionine is positioned immediately before the M domain of CiAG1. The additional N-terminal region apparently has no specific function since AG without N-terminal region was still shown to be functionally normal in vitro. AG homologous genes lacking the N-terminal extension have been identified in a few species such as LLAG1 from Lilium longiflorum (Benedito et al., 2004), PrAG1 from Pinus radiata (Liu, 2012), and HpAG from H. plantaginea (Wang et al., 2012).

Among the three cultivars, the maturation time of the staminate and pistillate flowers are different; qRT-PCR analysis demonstrated that CiAG may not be a reason to the heterodichogamy of flowers. Expression patterns of all the three cultivars indicated that CiAG was mainly expressed in reproductive tissues; however, a weak expression in vegetative tissues was also observed. The expression pattern of CiAG is similar to PTAG from poplar (Brunner et al., 2000). It is reported that the AG-lineage genes are generally expressed in reproductive organs, not in the vegetative organs (Chaidamsari et al., 2006; Wu et al., 2004; Yanofsky et al., 1990). This study suggests that AG may participate in regulation of vegetative phase, and the diversity functions of AG gene may exist in different plant species during evolution.

To further analyse the function of CiAG, constitutive overexpression of CiAG was undertaken in Arabidopsis. CiAG gene induced early flowering, reduced plant height and curled leaves, and was able to suppress floral A function in the second whorl in transgenic plants. Ectopic expression of AG orthologs in model plants showed transitions of sepals into carpel-like structures, and petals to filament-like structures (Chaidamsari et al., 2006; Kempin et al., 1993; Pnueli et al., 1994; Rigola et al., 2001), while there are reports that sepals showed almost no obvious conversion into carpel structures (Causier et al., 2002; Wu et al., 2012). In 35S::CiAG transgenic Arabidopsis, flowers homeotically transformed petals to filament-like structures, whereas sepals showed no obvious phenotypic changes. As for the ag-like flowers in T2 transgenic Arabidopsis, the reason may be the cosuppression of CiAG with endogenous AG in Arabidopsis (Napoli et al., 1990) resulting in posttranscriptional gene silencing. This indirectly demonstrates that CiAG is a homologous gene of AMAGOUS.

In the regulatory networks of flower development, AG interacted with multiple genes. AP1 does not inhibit the expression of AG gene (Busch et al., 1999; Werner et al., 1999). But, AP2 and other five genes in Arabidopsis, LEUNIG (LUG), CURLYLEAF (CLF), STERILE APETALA (SAP), ANT EGUIMENTA (ANT) and SEUSS (SEU), negatively regulate AG expression in the first and second whorls (Byzova et al., 1999; Liu and Meyerowitz, 1995). The expression of AG in stamen and pistil is inhibited by AP2 and LEUNIG (LUG) (Deyholos and Sieburth, 2000; Franks et al., 2002). LEY and WUSCHEL (WUS) genes are involved in accurate activation of AG expression in the third and fourth whorls, whereas AG negatively regulates WUS in the late stage of floral organ to end-flower development (Busch et al., 1999; Xu and Chang, 2005). Pecan is a monoecious and allogamous plant. The male and female flowering is not consistent in different varieties. From the result, we can see that CiAG gene was strongly expressed in reproductive organs and CiAG gene overexpression in Arabidopsis could make it flower earlier. It

Fig. 4. Detection of CiAG gene in transgenic and wide-type Arabidopsis. A: Products of RT-PCR with primers specific for CiAG; B: Products amplified with 18S-specific primers, used as control. WT, wild-type plants; L1–5, transgenic lines.

Fig. 5. Floral and vegetative morphology of transgenic and wide-type Arabidopsis. (A) Wild-type Arabidopsis plant (left) and transgenic plants at the same developmental stage over-expressing CiAG (right). (B) Early flowering of 35S::CiAG transgenic plants with four rosette leaves. (C) Flower bud of wild type at developmental stage 11 (Smyth et al., 1990). (D) Flower buds of transgenic plants at the developmental stage 11. (E) Sepals of transgenic lines could not completely enclose the developing buds. (F) Stamens of transgenic lines extended above sepals and petals. (G) Stamens of wild type were shorter than petals. (H) The base of the petal was converted into filament-like structure in 35S::CiAG transgenic plants. (I) Sepals were curled in 35S::CiAG transgenic plants. (J) ag-like flowers. (K) A: Products of RT-PCR with primers specific for CiAG and endogenous AG (Arabidopsis AG gene); B: Products amplified with 18S-specific primers, used as control. WT, wild-type plants; L1–3, ag-like plants.
shortened the juvenile phase and promoted the development of stamens. So we speculate that CiAG may be helpful for yield in pecan. However, the mechanism of CiAG gene function is complex and needs to be further studied.

**Literature Cited**


