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 illinoensis* (Wangenh.) 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 formation is controlled by A and B functions, stamen development is regulated by the combination of B and C functions, and carpel formation is determined by C function alone. It should be noted that there exists an antagonistic interaction between A and C functions. With the isolation of D function for ovule development (Angenent et al., 1995) and E function (Theissen, 2001), which is required for the specification of petals, stamens, and carpels, the ABC model has been extended to the ABCDE model. All the genes belonging to the ABCDE model are members of the MADS-box gene family except for *APETALA2* (*AP2*) genes (Theissen et al., 2000). MADS-box genes encoding homeotic transcription factors are a highly conserved gene family and these genes widely exist in

fungi, plants, and animals (Alvarez-Buylla et al., 2000; Bodt et al., 2003; García-Maroto et al., 2003). All the ABCDE MADS-box genes are of the MIKC type with regard to the presence of four distinct domains, which are a highly conserved MADS-box (M) domain, an intervening (I) domain, a moderately conserved keratin (K) domain, and a C-terminal (C) domain (Theissen et al., 2000).

AG, a class C gene, is required for normal development of third and fourth whorl floral organs. In the third whorl, *AG* functions in the specification of stamens; in the fourth whorl, *AG* is required for specification of carpels and provision of determinacy to the floral meristem (Martin et al., 2006; Sieburth et al., 1995).

Ectopic expression of *GmGAL2* (*Glycine max AGAMOUS Like 2*) in *Arabidopsis* enhances flowering, under both long-day and short-day conditions, by promoting the expression of key flowering genes, *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*), and lowering the expression of floral inhibitor *FLOWERING LOCUS C* (*FLC*) (Xu et al., 2010). *HpAG*, a *Hosta plantaginea* Aschers *AGAMOUS* homologous gene, isolated from developing flowers, plays a crucial role in stamen specification and gynoecium development (Wang et al., 2012). Overexpression of a *Brassica rapa* MADS-box gene, *BrAGL20*, induces early flowering time phenotypes in *Brassica napus* (Hong et al., 2013). Two transcript isoforms of *AGAMOUS* homologues, *PrseAG* (*Prunus serrulata AGAMOUS*) from single and *PrseAG-I* from double flower *Prunus lannesiana*, respectively, showed different functions. The transgenic *Arabidopsis* containing 35S::

PrseAG displayed extremely early flowering, bigger stamens and carpels, and homeotic conversion of petals into staminoid organs, but ectopic expression of *PrseAG-I* could not mimic the phenotypic ectopic expression of *PrseAG* in *Arabidopsis* (Liu et al., 2013). The legume *Medicago truncatula* contains three C-lineage genes in its genome: two euAG genes (*MtAGa* and *MtAGb*) and one PLENA-like gene (*MtSHP*). *MtAGa* and *MtAGb* were expressed early in the floral meristem, and in the third and fourth floral whorls during floral development. In contrast, *MtSHP* expression appears late during floral development (Serwatowska et al., 2014). On the basis of the highly conserved domain of MADS, homologues of *AG* have allowed cloning from a number of species (Zahn et al., 2006) such as apple (Van der Linden et al., 2002), grapevine (Boss et al., 2001), black cherry (Liu et al., 2010), and Japanese apricot (Hou et al., 2011).

Pecan (*C. illinoensis*), which belongs to the Juglandaceae family, is of great nutritional value (Hal, 2000). It originates from the northern United States and has been introduced in China for ≈100 years. The flower of pecan is unisexual; staminate flower consists of a calyx and androecium, whereas the pistillate flower has a calyx and a pistil; both of them have no petals. The cultivars of pecan can be divided into two categories: protogynous, such as ‘Mahan’, ‘Kanza’, and ‘Posey’; and protandrous such as ‘Pawnee’, ‘Osage’, and ‘Canton’ (Reid and Hunt, 2000; Wood et al., 1997). As 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 adaptor primer using PrimeScript RTase (TaKaRa, Japan).

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We have obtained eleven highly conserved MADS domain amino acid motifs from pecan previously. In this study, we cloned the full-length cDNA of the fragment 1 which belonged to *AGAMOUS* group (Mo et al., 2013). The gene-specific primers GSP1 (5'-CACTAC TAATCGTCAAGTCACCTTCTGT-3') and GSP2 (5'-CTTCTGTAAGAGGCGCAA CGGCTT-3') were designed based on the conserved MADS domain nucleotide sequence. Nested PCR was carried out to isolate the 3' end of the *CiAG* gene. The gene-specific primer GSP1 and an abridged universal amplification primer AUAP (5'-GGCCACGCGTGCAGTAGTAC-3') were used for first-round PCR, and the gene-specific primer GSP2 (5'-CTTCTGTAA GAGGCGCAACGGCTT-3') and AUAP 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'-CGCCATAACAGGGCAA TAACCT-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* (ACH72974); *Jatropha curcas*, *JcAG* (AEA11211); *Gossypium hirsutum*, *GhMADS5* (ABM69043); *Petunia xhybrida*, *FBP7* (CAA57311) and *FBP11* (CAA57445); *Arabidopsis thaliana*, *AGAMOUS* (NP_567569), *API* (NP_177074), *CAL* (Q39081), *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'-AGGCTGCTACTCTACGACAAC-3' and QTAG-R: 5'-GGTTCCTCTCATTCTCCGC TATC-3'. The *CiActin* gene was used as a positive internal control with primers ACTIN-F (5'-GCTGAACGGGAAATTGTC-3'), and ACTIN-R (5'-AGAGATGGCTGGAAGAGG-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); *Carya illinoensis*, *CiAG*; *Juglans regia*, *JrAG* (CAC38764); *Gossypium hirsutum*, *GhMADS5* (ABM69043); *Prunus persica*, *PpMADS4* (AAU29513); *Momordica charantia*, *McMADS2* (ABC25564); *Citrus sinensis*, *CsAG* (ADP02394); *Prunus serotina*, *PsAG* (ACH72974); *Jatropha curcas*, *JcAG* (AEA11211).

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 $2^{-\Delta\Delta Ct}$ 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 *Bam*HI 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* were 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. illinoensis*, 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 *ChMADS1* 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 fruitlets) of the three cultivars (Mahan, Pawnee, and Shaoling). 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).

Fig. 2. Phylogenetic analysis of *AG*-like MADS-box proteins. *CiAG* from pecan is boxed. *Prunus persica*, PpMADS4 (AAU29513); *Prunus serotina*, PsAG (ACH72974); *Momordica charantia*, McMADS2 (ABC25564); *Jatropha curcas*, JcAG (AEA11211); *Corylus heterophylla*, ChMADS1 (AEU08497); *Carya illinoensis*, *CiAG*; *Juglans regia*, JrAG (CAC38764); *Citrus sinensis*, CsAG (ADP02394); *Arabidopsis thaliana*, AGAMOUS (NP_567569), AGL11 (NP_192734); *Gossypium hirsutum*, GhMADS5 (ABM69043); *Petunia xhybrida*, FBP7 (CAA57311), FBP11 (CAA57445); SEP4 (NP_849930); SEP3 (NP_850953); SEP1 (NP_568322); SEP2 (NP_186880); AGL79 (NP_189645); AGL8/FUL (NP_568929); AP1 (NP_177074); CAL (Q39081); PI (NP_197524); AP3 (NP_191002).

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 plants and seven homozygous T3 plants displayed floral homeotic changes and were further analyzed.

In general, transgenic *Arabidopsis* flowered earlier (fourth rosette leaf appeared) than wild-type plants, displayed reduced height, small and curled rosette leaves, and cauline leaves (Fig. 5A and B). In wild-type

plants at stage 11 (Fig. 5C), flower buds were enclosed, when stigmatic papillae just appeared and the stigma had not extended above the buds (Smyth et al., 1990); however, the stigma of transgenic lines had already reached above the sepals (Fig. 5D). Since sepals could not completely enclose the developing buds (Fig. 5E), 35S::*CiAG* plants showed prematurely open floral buds. In transgenic plants, long stamens extended above sepals and petals (Fig. 5F); in contrast, the stamens of wild plants were shorter than petals (Fig. 5G). In the late-developing flowers of transgenic lines, the organs of the second whorl was homeotically changed, the bases of the petals were thin and transformed homeotically into filament-like structures (Fig. 5H), whereas the sepals were curled and had no obvious homeotic changes

Fig. 3. Expression pattern of *CiAG* gene revealed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis. Branches-CG, current-growth branches; Flowers-P, pistillate flowers; Flowers-S, staminate flowers.

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.

(Fig. 5I). Unexpectedly, three of the T2 transgenic plants have *ag*-like flowers (Fig. 5J); RT-PCR with primers for *CiAG* crossing with the endogenous *AG* (*Arabidopsis AG* gene) indicated the transcription of *AG* and *CiAG* genes (Fig. 5K).

Discussion

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; Wagner et al., 1999). But, *AP2* and other five genes in *Arabidopsis*, *LEUNIG* (*LUG*), *CURLYLEAF* (*CLF*), *STERILE APETALA* (*SAP*), *ANT EGUMENTA* (*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 Chong, 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

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

- Alvarez-Buylla, E.R., S. Pelaz, S.J. Liljegren, S.E. Gold, C. Burgeff, G.S. Ditta, L. Ribas de Pouplana, L. Martínez-Castilla, and M.F. Yanofsky. 2000. An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. *Proc. Natl. Acad. Sci. USA* 97:5328–5333.
- Angenent, G.C., J. Franken, M. Busscher, A. van Dijken, J.L. van Went, H.J. Dons, and A.J. van Tunen. 1995. A novel class of MADS box genes is involved in ovule development in petunia. *Plant Cell* 7:1569–1582.
- Benedito, V.A., P.B. Visser, J.M. van Tuyl, G.C. Angenent, S.C. De Vries, and F.A. Krens. 2004. Ectopic expression of *LLAG1*, an *AGAMOUS* homologue from lily (*Lilium longiflorum* Thunb.) causes floral homeotic modifications in *Arabidopsis*. *J. Expt. Bot.* 55:1391–1399.
- Bodt, S.D., R.Y.V.D. Peer, and G. Theibsen. 2003. And then there were many: MADS goes genomic. *Trends Plant Sci.* 8:475–483.
- Boss, P.K., M. Vivier, S. Matsumoto, I.B. Dry, and M.R. Thomas. 2001. A cDNA from grapevine (*Vitis vinifera* L.), which shows homology to *AGAMOUS* and *SHATTERPROOF*, is not only expressed in flowers but also throughout berry development. *Plant Mol. Biol.* 45:541–553.
- Brunner, A.M., W.H. Rottmann, L.A. Sheppard, K. Krutovskii, S.P. DiFazio, S. Leonardi, and S.H. Strauss. 2000. Structure and expression of duplicate *AGAMOUS* orthologues in poplar. *Plant Mol. Biol.* 44:619–634.
- Busch, M.A., K. Bomblies, and D. Weigel. 1999. Activation of a floral homeotic gene in *Arabidopsis*. *Science* 285:585–587.
- Byzova, M.V., J. Franken, M.G. Aarts, J. de Almeida-Engler, G. Engler, C. Mariani, M.M. Van Lookeren Campagne, and G.C. Angenent. 1999. *Arabidopsis* *STERILE APETALA*, a multifunctional gene regulating inflorescence, flower, and ovule development. *Genes Dev.* 13:1002–1014.
- Causier, B., M. Kieffer, and B. Davies. 2002. MADS-box genes reach maturity. *Science* 296:275–276.
- Chaidamsari, T., Samanhudi, H. Sugiarti, D. Santoso, G.C. Angenent, and R.A. de Maagd. 2006. Isolation and characterization of an *AGAMOUS* homologue from cocoa. *Plant Sci.* 170:968–975.
- Clough, S.J. and A.F. Bent. 1998. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735–743.
- Coen, E.S. and E.M. Meyerowitz. 1991. The war of the whorls: Genetic interactions controlling flower development. *Nature* 353:31–37.
- Deyholos, M.K. and L.E. Sieburth. 2000. Separable whorl-specific expression and negative regulation by enhancer elements within the *AGAMOUS* second intron. *Plant Cell* 12:1799–1810.
- Franks, R.G., C. Wang, J.Z. Levin, and Z. Liu. 2002. *SEUSS*, a member of a novel family of plant regulatory proteins, represses floral homeotic gene expression with *LEUNIG*. *Development* 129:253–263.
- García-Maroto, F., M.-J. Carmona, J.-A. Garrido, M. Vilches-Ferrón, J. Rodríguez-Ruiz, and D. López Alonso. 2003. New roles for MADS-box genes in higher plants. *Biol. Plant.* 46:321–330.
- Hal, G.D. 2000. Pecan food potential in prehistoric North America. *Econ. Bot.* 54:103–112.
- Hong, J.K., S.Y. Kim, K.S. Kim, S.J. Kwon, J.S. Kim, J.A. Kim, S.I. Lee, and Y.H. Lee. 2013. Overexpression of a *Brassica rapa* MADS-box gene, *BrAGL20*, induces early flowering time phenotypes in *Brassica napus*. *Plant Biotechnol. Rep.* 7:231–237.
- Hou, J.H., Z.H. Gao, Z. Zhang, S.M. Chen, T. Ando, J.Y. Zhang, and X.W. Wang. 2011. Isolation and characterization of an *AGAMOUS* homologue *PmAG* from the Japanese apricot (*Prunus mume* Sieb. et Zucc.). *Plant Mol. Biol. Rpt.* 29:473–480.
- Kempin, S.A., M.A. Mandel, and M.F. Yanofsky. 1993. Conversion of perianth into reproductive organs by ectopic expression of the tobacco floral homeotic gene *NAG1*. *Plant Physiol.* 103:1041–1046.
- Kramer, E.M., M.A. Jaramillo, and V.S. Di Stilio. 2004. Patterns of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. *Genetics* 166:1011–1023.
- Liu, J.-J. 2012. Ectopic expression of a truncated *Pinus radiata* *AGAMOUS* homolog (*PrAG1*) causes alteration of inflorescence architecture and male sterility in *Nicotiana tabacum*. *Mol. Breed.* 30:453–467.
- Liu, X., J.M. Anderson, and P.M. Pijut. 2010. Cloning and characterization of *Prunus serotina* *AGAMOUS*, a putative flower homeotic gene. *Plant Mol. Biol. Rpt.* 28:193–203.
- Liu, Z. and E.M. Meyerowitz. 1995. *LEUNIG* regulates *AGAMOUS* expression in *Arabidopsis* flowers. *Development* 121:975–991.
- Liu, Z.X., D.D. Zhang, D. Liu, F.L. Li, and H. Lu. 2013. Exon skipping of *AGAMOUS* homolog *PrseAG* in developing double flowers of *Prunus lannesiana* (Rosaceae). *Plant Cell Rpt.* 32:227–237.
- Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* 25:402–408.
- Martin, T., M. Hu, H. Labbe, S. McHugh, A. Svircev, and B. Miki. 2006. *PpAG1*, a homolog of *AGAMOUS*, expressed in developing peach flowers and fruit. *Can. J. Bot.* 84:767–776.
- Mo, Z.H., J.Y. Zhang, M. Zhai, X.D. Jia, J.P. Xuan, Y.R. Li, and Z.R. Guo. 2013. Cloning and sequence analysis of MADS-box gene conservative fragment from pecan (*Carya illinoensis*). *J. Southern Agr.* 44:730–734.
- Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* 4:279–289.
- Pnueli, L., D. Hareven, S.D. Rounsley, M.F. Yanofsky, and E. Lifschitz. 1994. Isolation of the tomato *AGAMOUS* gene *TAG1* and analysis of its homeotic role in transgenic plants. *Plant Cell* 6:163–173.
- Reid, W. and K.L. Hunt. 2000. Pecan production in the northern United States. *HortTechnology* 10:298–301.
- Rigola, D., M.E. Pè, L. Mizzi, F. Ciampolini, and M. Sari-Gorla. 2001. *CaMADS1*, an *AGAMOUS* homologue from hazelnut, produces floral homeotic conversion when expressed in *Arabidopsis*. *Sex. Plant Reprod.* 13:185–191.
- Serwatowska, J., E. Roque, C. Gomez-Mena, G.D. Constantin, J. Wen, K.S. Mysore, O.S. Lund, E. Johansen, J.P. Beltran, and L.A. Canas. 2014. Two eu*AGAMOUS* genes control C-function in *Medicago truncatula*. *PLoS One* 9:e103770.
- Sieburth, L.E., M.P. Running, and E.M. Meyerowitz. 1995. Genetic separation of third and fourth whorl functions of *AGAMOUS*. *Plant Cell* 7:1249–1258.
- Smyth, D.R., J.L. Bowman, and E.M. Meyerowitz. 1990. Early flower development in *Arabidopsis*. *Plant Cell* 2:755–767.
- Theissen, G. 2001. Development of floral organ identity: Stories from the MADS house. *Curr. Opin. Plant Biol.* 4:75–85.
- Theissen, G., A. Becker, A.D. Rosa, A. Kanno, J.T. Kim, T. Münster, K.-U. Winter, and H. Saedler. 2000. A short history of MADS-box genes in plants. *Plant Mol. Biol.* 42:115–149.
- Van der Linden, C.G., B. Vosman, and M.J.M. Smulders. 2002. Cloning and characterization of four apple MADS box genes isolated from vegetative tissue. *J. Expt. Bot.* 53:1025–1036.
- Wagner, D., R.W. Sablowski, and E.M. Meyerowitz. 1999. Transcriptional activation of *APETALA1* by *LEAFY*. *Science* 285:582–584.
- Wang, Y., X.M. Zhang, Z.X. Liu, D.D. Zhang, J.Z. Wang, D. Liu, F.L. Li, and H. Lu. 2012. Isolation and characterization of an *AGAMOUS*-like gene from *Hosta plantaginea*. *Mol. Biol. Rpt.* 39:2875–2881.
- Wood, B.W., M.W. Smith, R.E. Worley, P.C. Anderson, T.T. Thompson, and L.J. Grauke. 1997. Reproductive and vegetative characteristics of pecan cultivars. *HortScience* 32:1028–1033.
- Wu, F., Y. Xu, F.Q. Chang, X.D. Li, and R.C. Ma. 2004. Cloning and characterization of two MADS box genes from peach (*Prunus persica*). *Yi Chuan Xue Bao.* 31:908–918.
- Wu, W., F. Chen, D. Jing, Z. Liu, and L. Ma. 2012. Isolation and characterization of an *AGAMOUS*-like gene from *Magnolia wufengensis* (Magnoliaceae). *Plant Mol. Biol. Rpt.* 30:690–698.
- Xu, J.H., X.F. Zhong, Q.Z. Zhang, and H.Y. Li. 2010. Overexpression of the *GmGAL2* gene accelerates flowering in *Arabidopsis*. *Plant Mol. Biol. Rpt.* 28:704–711.
- Xu, Y. and K. Chong. 2005. Progress in research on plant stem cell organizer gene *WUSCHEL*. *J. Plant Physiol. Mol. Biol.* 31:461–468.
- Yanofsky, M.F., H. Ma, J.L. Bowman, G.N. Drews, K.A. Feldmann, and E.M. Meyerowitz. 1990. The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature* 346:35–39.
- Zahn, L.M., J.H. Leebens-Mack, J.M. Arrington, Y. Hu, L.L. Landherr, C.W. Depamphilis, A. Becker, G. Theissen, and H. Ma. 2006. Conservation and divergence in the *AGAMOUS* subfamily of MADS-box genes: Evidence of independent sub- and neofunctionalization events. *Evol. Dev.* 8:30–45.