

# Isolation and Characterization of an *AGAMOUS* Homolog from *Fraxinus pennsylvanica*

Ningxia Du · Paula M. Pijut

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**Abstract** An *AGAMOUS* homolog (*FpAG*) was isolated from green ash (*Fraxinus pennsylvanica*) using a reverse transcriptase polymerase chain reaction method. Southern blot analysis indicated that *FpAG* was present as a single-copy sequence in the genome of green ash. RNA accumulated in the reproductive tissues (female inflorescence, male inflorescence, and fruit) and vegetative tissues (leaves and in vitro-germinated seedlings). Expression was higher in reproductive tissues than in vegetative tissues. Ectopic expression of *FpAG* in transgenic *Arabidopsis* plants resulted in a range of weak to strong *APETALA2* (*AP2*) mutant-like phenotypes, including early flowering, curly leaves, and conversion of petals to stamens. These data indicate functional homology between *FpAG* and *AGAMOUS*.

**Keywords** *Arabidopsis* · MADS-box · Flower development · Green ash

## Introduction

Green ash (*Fraxinus pennsylvanica*; Oleaceae; Section *Melioides*) is a widely distributed native tree species,

planted for timber production and popular for landscaping in North America. Green ash is dioecious and diploid, and its inconspicuous flowers are apetalous, occur in inflorescences, and appear with or just before the leaves elongate in spring (Remphrey 1989; Wallander 2008). Female ash flowers, in the section *Melioides*, consist of a calyx and one pistil, and male flowers have two stamens and a small calyx (Wallander 2008). The fruit is a one-seeded samara borne in clusters.

Fertility of transgenic trees may have an effect on the rate of gene flow from transgenic trees to the wild (Brunner et al. 2007). This raises a particular important issue in bio-safety when transgenic plants are released, especially for tree species, because of the fact that trees have undergone less domestication. Genetically engineered reproductive sterility might provide both genetic containment and an increase in growth rates for transgenic trees (Strauss et al. 1995). In an effort to reduce concerns for transgenic plant release, we are working to develop reproductively sterile green ash through manipulation of flower development genes; *AGAMOUS* (*AG*) is one of those genes. *AG* is a C-function floral organ identity gene found in *Arabidopsis* and responsible for identifying reproductive organs and required for determinacy (Bowman et al. 1989; Yanofsky et al. 1990). Sequence conservation of this gene among flowering plants has allowed isolation of homologs from a number of species (Yanofsky 1995). Sequence conservation of *AG* should facilitate the cloning of an *AG* homolog from green ash.

In most flowering plants, the floral organ arrangement is in four whorls, including sepals, petals, stamens, and carpels. The ABC model of flower development explains the specification of floral organ identity (Coen and Meyerowitz 1991). Class A genes control the formation of sepals in whorl 1, while both class A and B genes are

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N. Du  
Purdue University, Department of Forestry and Natural Resources,  
Hardwood Tree Improvement and Regeneration Center (HTIRC),  
715 West State Street,  
West Lafayette, IN 47907, USA

P. M. Pijut (✉)  
USDA Forest Service, Northern Research Station, HTIRC,  
715 West State Street,  
West Lafayette, IN 47907, USA  
e-mail: ppijut@purdue.edu  
e-mail: ppijut@fs.fed.us

required for the formation of petals in whorl 2. Class C genes (e.g., *AG*) control the formation of carpels in whorl 4, while both B- and C-class genes are required for the formation of stamens in whorl 3. Coexpression of the floral binding protein (*FBP7*) and *FBP11* in petunia (*Petunia hybrida*) revealed the D-function gene involved in ovule development (Angenent et al. 1995). Additional studies on MADS-box genes revealed a fifth class of genes, E-function, specifying petal, stamen and carpel, and possibly ovule identity (Theiben 2001). The A, B, and C genes belong to the MADS-box family of transcription factors, which are highly conserved in plants, animals, and yeast (Shore and Sharrocks 1995). Except for *APETALA2* (*AP2*) of class A, all genes for flower development in the ABC model are MICK-type MADS-box genes because of the presence of four distinct domains, which are M (MADS), I (intervening), K (keratin-like), and C (C terminal) (Theissen et al. 2000). In *Arabidopsis*, recessive mutants for *AG* show petals instead of stamens in whorl 3 and a new flower instead of carpels in whorl 4, giving rise to indeterminacy of the flower, which is patterned as (4 sepals, 4 petals, 6 petals)<sub>n</sub>. Overexpression of *AG* in *Arabidopsis* induced homeotic conversion of sepals to carpels and petals to stamens (*AP2* mutant phenotype) (Mizukami and Ma 1992) in flowers in a manner similar to mutations produced via loss of function in a class A gene, as a result of antagonistic interaction between A and C functions (Coen and Meyerowitz 1991). *AG* homologs have been isolated from several species such as petunia (Tsuchimoto et al. 1993), tomato (Pnueli et al. 1994), tobacco (Kempin et al. 1993), poplar (Brunner et al. 2000), black spruce (Rutledge et al. 1998), and sweetgum (Liu et al. 1999). Constitutive overexpression of *AG* was used to investigate gene function.

In this study, an *AG* homolog (*FpAG*) was isolated from flowers of green ash. Sequence analysis, expression analysis, as well as ectopic expression in transgenic *Arabidopsis* plants suggest that *FpAG* was a functional *AG* homolog in green ash.

## Materials and Methods

### Plant Materials

Female and male inflorescences, leaves, and fruits of green ash (*F. pennsylvanica*) were collected in July 2007 and April 2008 from a mature tree on the campus of Purdue University, West Lafayette, IN, USA, for RNA extraction. In vitro-germinated seedlings, including roots, stems, cotyledons, and young leaves, of green ash were also collected for RNA extraction. Samples were frozen in liquid nitrogen immediately after collection and stored at  $-80^{\circ}\text{C}$  until used.

### Nucleic Acid Extraction and PCR Cloning of Target Gene

Total RNA extraction from green ash tissues was according to the method of Kolosova et al. (2004). RNA was re-suspended in 0.1% diethylpyrocarbonate-treated water, followed by DNase treatment. The isolation of genomic DNA from green ash leaves was performed as described by LeFort and Douglas (1999).

A reverse transcriptase polymerase chain reaction (RT-PCR) based strategy was used for cloning. Degenerate primers AGP1 [5'-GGA TCGA(A/G)AACAC(A/C/G)ACA(A/C/G/T)A(C/T)CG-3'] and AGP2 [5'-G(C/T)(C/T)TCCTG(C/T)TGGTA(A/G)(A/T)ACTG-3'] were designed based on a total of 17 *AG* homologous nucleotide sequences (accession no. AAC06238, ABA39727, AAT46102, CAA86585, AAZ77747, AAL92522, ABU50335, AAA17033, ABN46892, AAC08528, AAD01744, CAA16753, AAA34197, CAC80858, AAD00025, AAY63868, and AAR98731) retrieved from the GenBank database, and the nucleic acid sequence and deduced amino acid sequences were aligned using ClustalW. The primers were used to amplify an internal fragment spanning part of the MADS domain and part of the K domain. First-strand cDNA was synthesized from 2  $\mu\text{g}$  total RNA from male inflorescences with dT23VN as primer using Protoscript First-Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA). Using 2  $\mu\text{l}$  out of 20  $\mu\text{l}$  of the cDNA synthesis product, a 20- $\mu\text{l}$  PCR reaction was prepared containing 2.5  $\mu\text{l}$  10 $\times$  PCR buffer (Invitrogen, Carlsbad, CA, USA), 1  $\mu\text{l}$  10 mM dNTP, 0.8  $\mu\text{l}$  50 mM  $\text{Mg}^{2+}$ , 1  $\mu\text{l}$  10  $\mu\text{M}$  AGP1 and AGP2 primers, respectively, and 0.2  $\mu\text{l}$  of 5 U  $\mu\text{l}^{-1}$  Taq polymerase. The cycling program consisted of an initial denaturation at  $94^{\circ}\text{C}$  for 2 min, followed by five cycles of  $94^{\circ}\text{C}$  for 30 s,  $42^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 1 min, 35 additional cycles of  $94^{\circ}\text{C}$  for 30 s,  $47^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 1 min, and a final extension at  $72^{\circ}\text{C}$  for 10 min. Electrophoretic analysis showed amplification of a single band of expected size (252 bp) (data not shown), which was then cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and individual colonies collected for DNA sequence analysis using a BigDye Terminator v3.0 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Nested PCR for both 5' rapid amplification of cDNA ends (RACE) and 3' RACE (FirstChoice RLM-RACE; Applied Biosystems) were applied to clone 5' end and 3' end of target gene. For 5' RACE PCR, first-round PCR was conducted using AGP3 [5'-TTGAGGAATCTGAGCAGGCTTTC-3'] as gene-specific outer primer and a 5' RACE outer primer provided in kit; second-round PCR was conducted using AGP4 [5'-GACGACCTCGGGTAGAGAA GACA-3'] as gene-specific inner primer and 5' RACE inner primer provided in the kit to amplify first-round PCR product. For 3' RACE PCR, the AGP5 [5'-CCTTCTGTAAGCGCCGCAAC-3'] gene-specific outer primer and 3' RACE

outer primer were used for first-round PCR, and the AGP6 [5'-GCCTGCTCAGATTCCTCAAACAA-3'] gene-specific inner primer and 3' RACE inner primer were used for second-round PCR to amplify first-round PCR product. A-tailed 3' RACE product and 5' RACE product by A-tailing were cloned into pGEM-T Easy vector for DNA sequence analysis. Sequences were analyzed and assembled into contigs using SeqMan II software (DNASTar, Madison, WI, USA). To amplify a full-length cDNA (coding region from start to stop codons) of *FpAG*, 2  $\mu$ l of the first-strand cDNA from green ash male inflorescence was used as a template for PCR using AGP7 [5'-GCT CTAGAATGGCATTGCAGAGTGATCAA-3'] (covering the presumed start codon and creating an *Xba*I site) and AGP8 [5'-CCCGAGCTCTCAGACTAATTGAAGAGGTGG-3'] (covering the presumed stop codon and creating a *Sac*I site). The cycling program consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR product was used to produce an *Xba*I and *Sac*I fragment containing the *FpAG* coding region to replace the GUS coding region within the pBI121 binary vector, giving rise to pBI121-*FpAG*.

#### Expression Analysis

RT-PCR analysis was performed using Protoscript First-Strand cDNA Synthesis Kit (New England Biolabs). Five micrograms of total RNA extracted from reproductive tissues (male inflorescences, female inflorescences, and fruits) and vegetative tissues (leaves and in vitro-germinated seedlings) were used to synthesize first-strand cDNA using dT23VN as primer. For amplification of *FpAG* cDNA, 2  $\mu$ l out of 20  $\mu$ l of the cDNA synthesis product was used in a 20- $\mu$ l PCR reaction with *FpAG*-specific primers AGP7 and AGP8 as forward and reverse primers, respectively. The cycling program consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and concluding with a final extension at 72°C for 10 min. Twenty microliters of the RT-PCR was run in an agarose gel and photographed under UV light. As a control, 2  $\mu$ l of the first-strand RT reaction was used for amplification of part of 18S cDNA of green ash using 18SF [5'-AGAGGCCTACAA TGGTGGTG-3'] and 18SR [5'-CCTCCAATGGATCC TCGTTA-3'] as forward and reverse primers, respectively. The PCR conditions were as described for *FpAG*.

#### Southern Blot

For genomic DNA hybridization, green ash genomic DNA (20  $\mu$ g) from leaves was digested with *Eco*RI, *Hind*III, and *Xba*I (Invitrogen), separated by electrophoresis in a 0.8% (w/v) agarose gel, and blotted to a Hybond-N nylon

membrane (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The probe was the 3'-RACE fragment labeled with [ $\alpha$ -<sup>32</sup>P] dCTP Random Prime Labelling System (Rediprime II, GE Healthcare Bio-Sciences). Pre-hybridization and hybridization were carried out at 65°C overnight in buffer solution containing 6 $\times$  SSC containing 0.9 M NaCl and 0.09 M sodium citrate, 5 $\times$  Dehart's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 mg ml<sup>-1</sup> denatured salmon sperm DNA. The membrane was then washed at 65°C twice for 20 min in 2 $\times$  SSC, 0.1% SDS, and once for 10 min in 0.5 $\times$  SSC, 0.1% SDS. The membrane was exposed overnight to a Phosphor Imaging Plate (Fuji Photo Film Co., Tokyo, Japan) and developed in a scanner system.

#### Generation and Analysis of Arabidopsis Transformants

The plasmids pBI121-*FpAG* and pBI121 (negative control) were transformed into *Agrobacterium tumefaciens* strain EHA105 by heat shock (Hofgen and Willmitzer 1988) and then introduced into *Arabidopsis thaliana* ecotype Columbia (Col) by the floral dip method (Clough and Bent 1998). T<sub>1</sub> seeds were placed onto agar plates containing half-strength Murashige and Skoog medium (1962) with 50 mg l<sup>-1</sup> kanamycin as selection agent. The plates were incubated at 4°C for 3 days to break seed dormancy and then at 24°C in a culture room under long-day conditions (16 h light, 80  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>). Kanamycin-resistant T<sub>1</sub> seedlings were transferred to soil and kept at the same temperature and lighting conditions for analyzing flowering time and phenotype. Ectopic expression of *FpAG* in transgenic *Arabidopsis* was determined as described for RT-PCR analysis *FpAG* gene expression in different organs of green ash, but 25 cycles instead of 30 cycles were used. Gene-specific primer pairs AGP9 [5'-CATGCAAAAGAGGGAGATCAA-3'] and AGP10 [5'-GATCTTGCCGAGGGTAATCA-3'] were used to amplify the 3' end fragment of *FpAG* in six lines of first-generation transgenic *Arabidopsis*. Amplification of part of 18S cDNA of *Arabidopsis* was used as control.

## Results

#### Isolation and Sequence Analysis of *FpAG* cDNA from Green Ash

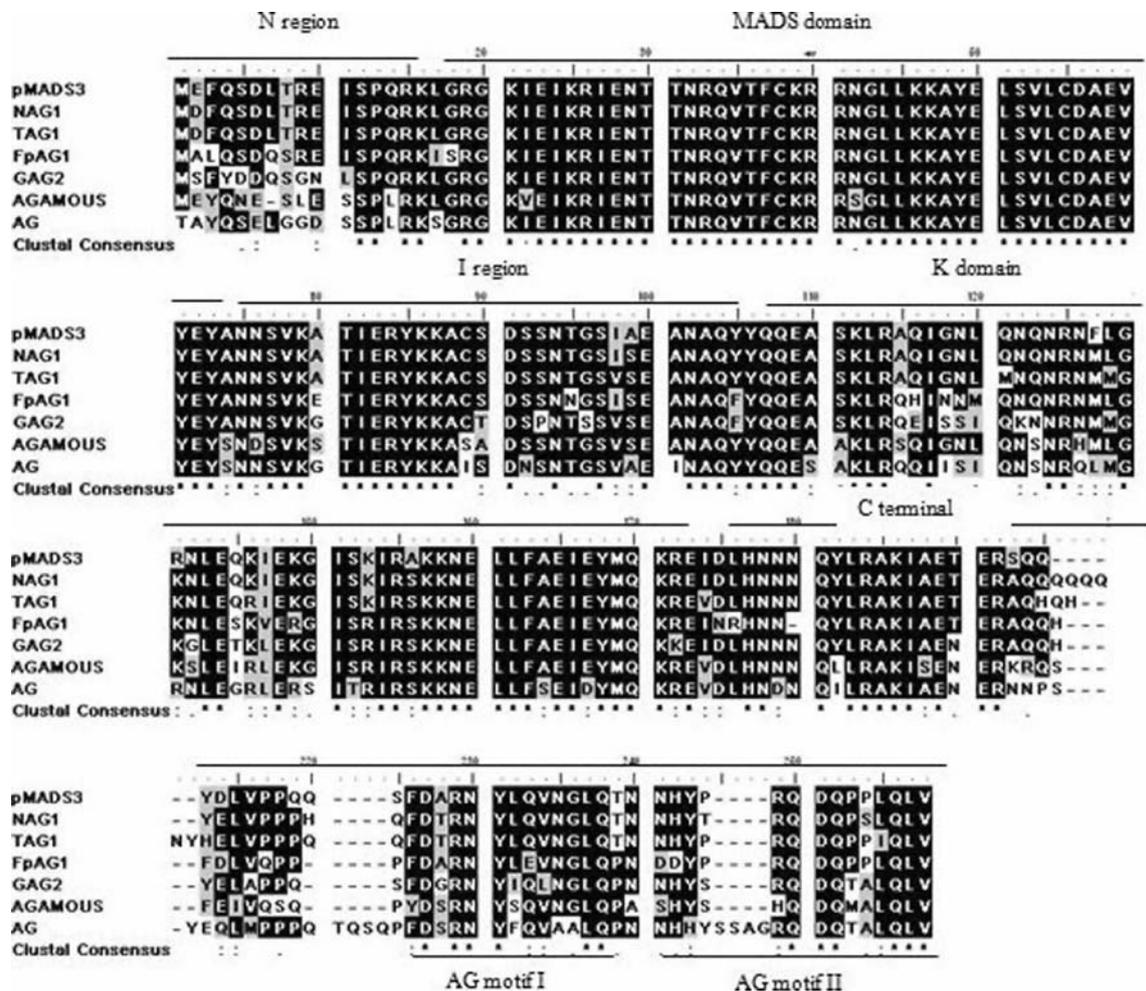
RT-PCR methods were used to isolate an *AG* homolog from green ash flower inflorescences (April) because the *AG* homologous gene was expected to be expressed at high levels at this stage. PCR amplification with degenerate primers was used to amplify a 252-bp internal fragment spanning part of the MADS domain and part of K domain to help ensure the identity of the resulting product. We isolated full-length cDNA of an *AG*-like gene from green

ash by the 5' RACE and 3' RACE method. The green ash cDNA of *FpAG* was 1,096 bp in length with a 90-bp 5' untranslated region and a 254-bp 3' untranslated region upstream of the poly(A) tail. *FpAG* encodes a putative protein of 242 amino acid residues. A comparison of deduced *FpAG* protein sequence with that of other *AG* homologs from GenBank database was generated (Fig. 1). The deduced amino acid sequence contains the 56 residue MADS domain, which was highly conserved among all of the other *AG* homologs described above. In addition to the conserved MADS domain, the *AG* homolog also possesses a K box domain, which was less conserved than the MADS domain and the C terminus. The alignment of *FpAG* with other *AG* amino acid sequences within the MADS-box domain revealed that there was only one non-conserved amino acid substitution in *FpAG*. *FpAG* shares 98% (55/56) amino acid similarity with pMADS3, an *AG* homolog from

*Petunia × hybrida*, *NAG1* from *Nicotiana tabacum*, *TAG1* from *Solanum lycopersicum*, *GAG2* from *Panax ginseng*, *AGAMOUS* from *Populus trichocarpa*, and *AG* from *A. thaliana*. As a whole, close homologs of *FpAG* are pMADS3 and *NAG1* (82% amino acid identity), followed by *TAG1* (79%), *GAG2* (78%), *AGAMOUS* (72%), and 67% with *Arabidopsis* *AG*. A comparison in the C terminal reveals several short amino acid conserved motifs among *FpAG* and other *AG* homologs (Fig. 1). Overall, sequence analysis showed that *FpAG* from green ash was the cognate homolog of *AG*.

### FpAG Expression Pattern

The spatial expression pattern of *FpAG* of green ash was analyzed by RT-PCR using primers designed to amplify the full-length cDNA of *FpAG* (729 bp) using various



**Fig. 1** Alignment of deduced amino acid sequences encoded by *FpAG* with *AG* homologs from *A. thaliana* (*AG*), *N. tabacum* (*NAG1*), *S. lycopersicum* (*TAG1*), *Petunia × hybrida* (*pMADS3*), *P. trichocarpa* (*Agamous*), and *P. ginseng* (*GAG2*). The alignment was generated by the ClustalW program and displayed with the Bioedit program. Identical amino acid residues in relation to *FpAG* are black and

conserved residues are in gray. Asterisks indicate identity with *FpAG*; dashes indicate gaps inserted to maximize alignment. The amino acid terminal extension (N), MADS, intervening (I), K, and carboxyl-terminal (C) domains are marked. The amino acid positions are shown on the top. *AG* motifs I and II, which are highly conserved regions reported by Kramer et al. (2004), are underlined

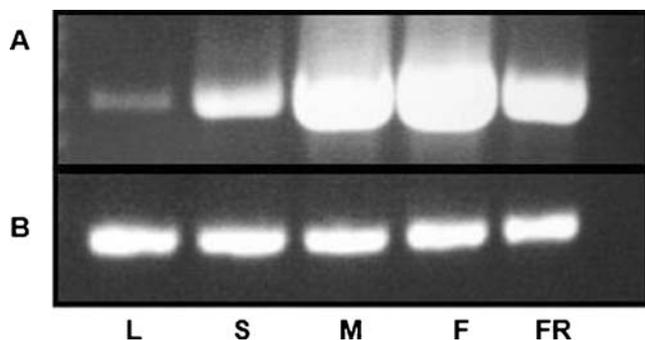
vegetative and reproductive tissues of green ash: leaf, in vitro-germinated seedlings, male and female inflorescences, and fruit. Amplification of an 18S fragment (220 bp) was used as a control. The results revealed that transcript of *FpAG* can be detected in both vegetative tissues (leaves and in vitro seedlings) and reproductive tissues (male, female inflorescences, and fruit) (Fig. 2). The intensity of bands also indicated that the amount of *FpAG* RNA in reproductive tissues was higher than in vegetative tissues, which corresponds with other C-class genes. Low levels of *FpAG* expression was also found in leaves from the mature tree and in vitro-germinated seeds including roots, stems, cotyledons, and young leaves.

#### Southern Blot

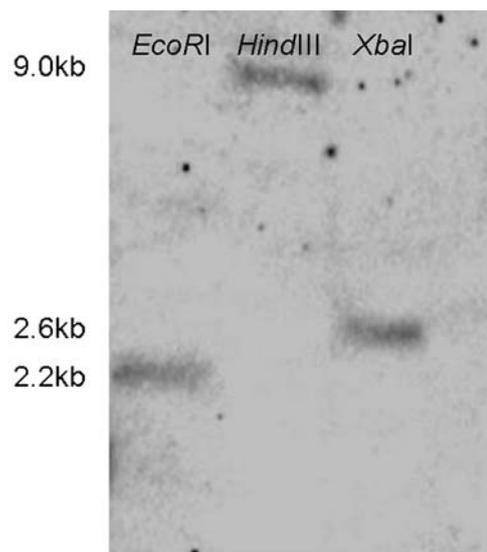
To ascertain whether *FpAG* was duplicated in green ash, a genomic DNA blot was performed using the 3' ends of *FpAG* as a probe. Results showed that *FpAG* was a single-copy gene in the green ash genome, because only one hybridization band was observed (Fig. 3).

#### Ectopic Expression of *FpAG* in Arabidopsis

Functional analysis of *FpAG* was further investigated by its ectopic expression in *Arabidopsis* to determine whether *FpAG* had the same function as *AG*. Transgenic *Arabidopsis* were obtained by *Agrobacterium*-mediated transformation using pBI121 binary vector containing the coding region of *FpAG* driven by the 35S promoter. Following transformation, transgenic seeds were selected on kanamycin-containing plates, and surviving plants were transferred to soil. Flowering time and the phenotypic alterations of transformed plants were analyzed in the T<sub>1</sub> generation. *FpAG*-expressing plants appeared to flower earlier (1 to 2 weeks) than plants transformed with the pBI121 empty vector. Twelve out of 25 independent kanamycin-resistant T<sub>1</sub>

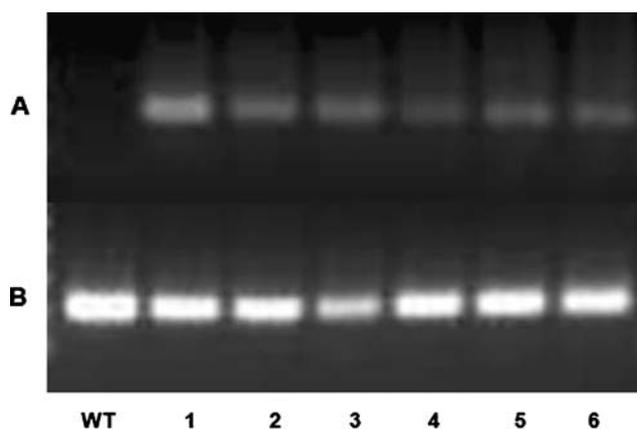


**Fig. 2** RT-PCR products from green ash RNA. **A** Products from *FpAG*-specific primers. **B** Products produced with 18S primers, used as a control. *L* leaf, *S* in vitro-germinated seedling, *M* male flower, *F* female flower, *FR* fruit



**Fig. 3** Southern blot analysis of *FpAG*. The genomic DNA extracted from green ash was digested with *EcoRI*, *HindIII*, or *XbaI*. Each lane contains 20 µg DNA

plants containing 35S::*FpAG* exhibited varying degrees of phenotypic alterations in vegetative and reproductive organs, in comparison to the wild type. Ectopic expression of *FpAG* was confirmed by RT-PCR analysis (203 bp) from six independent transformation events. *FpAG* was not expressed in wild-type plants, but was expressed in the transgenic plants (Fig. 4A). Transgenic lines T<sub>1</sub>-01, -02, and -03 showed higher expression of *FpAG* with stronger phenotypes than was seen with lines T<sub>1</sub>-04, -05, and -06, which had weak phenotypes. The accumulation levels of *FpAG* transcripts in different lines are consistent with phenotypic alterations.



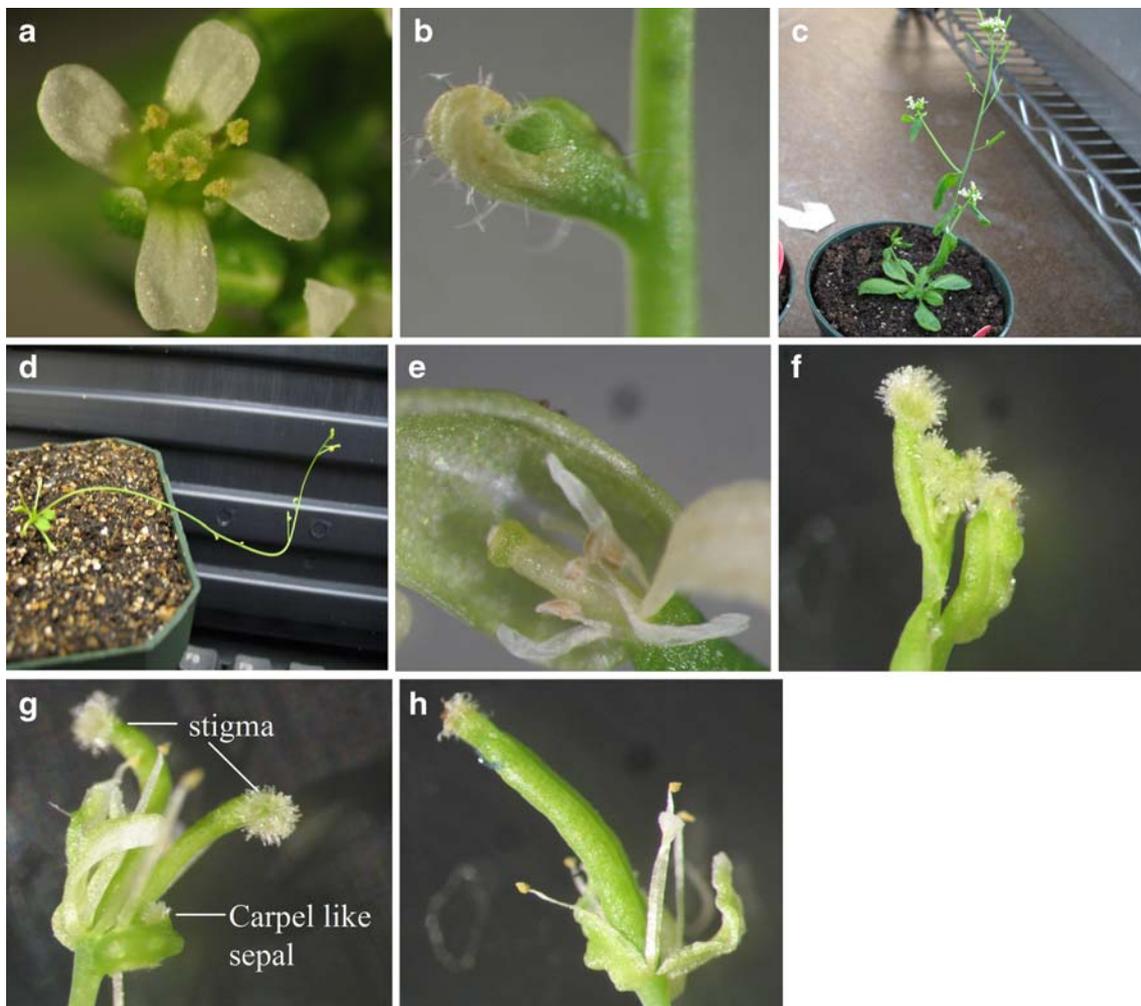
**Fig. 4** Overexpression of *FpAG* in *Arabidopsis*. **A** Products of RT-PCR with primers specific for 3' ends of *FpAG*. **B** Products amplified with 18S-specific primers, used as control (*WT* wild type; lanes 1–6 individual transgenic lines: T<sub>1</sub>-01, T<sub>1</sub>-02, T<sub>1</sub>-03, T<sub>1</sub>-04, T<sub>1</sub>-05, and T<sub>1</sub>-06)

According to the ABCDE model of flower development, transgenic plants overexpressing *AG* were expected to show *ap2*-mutant phenotype as the result of the interaction between A- and C-function genes. These phenotypic changes were divided into two categories: strong and weak *ap2*-like phenotypes. Strong *ap2*-like phenotypes showed reduced stature; small, curled cauline and rosette leaves; and loss of inflorescence determinacy (Fig. 5a–d) than the wild type. These plants also produced fewer flowers and seeds than the wild type (Fig. 5c, d). The most important characteristic of the transgenic plant with *AG* overexpression was the homeotic changes in the organs of the two outer floral whorls. Sepals were transformed into carpel-like structures (Fig. 5f), and the petals were thin and transformed into filament-like structures in some strong *ap2*-like plants (Fig. 5e, h). The most severe conversion

occurred in late-developing flowers. The most severely affected lines produced not only carpel-like structures, but also two pistils within one flower (Fig. 5g). Plants with weak *ap2*-like phenotype showed normal vegetative growth with fewer floral alterations; these presented only partly or completely developed petals. These observed phenotypic alterations suggest that the *FpAG* performs the same function as *AG*.

## Discussion

In this study, a RT-PCR approach utilizing degenerate primers spanning part of MADS-box and K domains to isolate an *AG* homolog from flower inflorescences of green ash was used. PCR-based cloning methods using degener-



**Fig. 5** Floral and vegetative morphology of *Arabidopsis*. **a** Wild-type flower with four sepals, four petals, six stamens, and pistil. **b** Transgenic lateral inflorescence entrapped in curled cauline leaf. **c** Rosette leaves, flower, and seed set of a wild-type plant. **d** Rosette leaves of a transgenic plant with a strong *ap2*-like phenotype. **e**

Transgenic flower with very thin petal. **f** Sepals converted into carpel-like structures. **g** Flower with two pistils and sepals that are converted into carpel-like structures. **h** Flower with curled sepals and petals converted into filament-like structures

ate primers were effective for isolating highly conserved genes (Li et al. 2000; Liu et al. 1999; Zhang et al. 2000). Identifying conserved regions of the sequences from a large range of organisms can increase the chance of obtaining the desired gene. Seventeen amino acid sequences of *AG* homolog were used to design degenerate primers and to successfully clone full-length cDNA of *FpAG*. The deduced amino acid sequence of *FpAG* revealed high homology with other *AG*-like sequences and also revealed that *FpAG* was a typical type II plant with MADS-box genes containing the MIKC structure. The deduced amino acid sequence contains the highly conserved 56 residue MADS-box domain, which was found mostly at the N terminus of proteins (Ma et al. 1991). It included a non-functional N terminal extension of the MADS-box since the truncated *AG* without this N terminal extension functioned normally in vitro (Huang et al. 1993; Pollock and Treisman 1991). However, not all *AG* homolog genes have this N terminal extension (Benedito et al. 2004; Kang et al. 1995; Li et al. 2002). Non-function of this extension might be the reason for its loss during evolution (Benedito et al. 2004). The K domain, which was located downstream of the MADS domain, was linked by a weakly conserved I domain to promote dimerization in plants (Riechmann and Meyerowitz 1997). The C terminus was also essential for *AG* function because transgenic plants expressing a truncated *AG* without the C terminus exhibited *AG* phenotype at a frequency of 50% (Mizukami et al. 1996). This indicates that C terminal of *AG* may function in some way which was not yet clear. Deduced amino acid alignment of C terminus revealed that it contains highly conserved motifs I and II (Fig. 1), which have been found in many *AG* homologs in angiosperms (Kramer et al. 2004) although the functions have not been ascertained.

RT-PCR analysis demonstrated that *FpAG* was expressed in floral tissue as well as leaves of the adult flowering tree and in vitro seedlings, with the highest expression levels in floral tissue and the least in vegetative tissues. Although expression of *AG* was not detected in wild-type *Arabidopsis* leaves, it has been reported that *CURLY LEAF* prevents *AG* expression in leaves during the vegetative phase (Goodrich et al. 1997). *FpAG* expression in vegetative tissue (leaves and in vitro seedlings) may be a result of less stringent repression controls in green ash, similar to *PTAG* vegetative (leaves) expression seen in poplar (Brunner et al. 2000) and *SAG1* vegetative (needles) expression in black spruce (Rutledge et al. 1998). Expression of an *AG* homolog in both floral tissue and vegetative tissue has important implications for regulating the expression of these genes. Directing cytotoxin expression using floral gene promoters may not only prevent the targeted floral tissues from completing development, but may also cause severe development and growth impairment in transgenic plants.

Skinner et al. (2000) found transgenic plants with significantly decreased growth when directing cytotoxin expression in transgenic poplar using floral promoters from tobacco and *Brassica*.

Because of a lack of genetic transformation protocols optimized for various plant tissues and genotypes (Du and Pijut 2009) and the long juvenile period before maturity of green ash, the functional analysis of *FpAG* was undertaken in the model species *Arabidopsis*. The ectopic expression of *FpAG* in transgenic *Arabidopsis* induced conversion of sepals to carpel-like structures, and petals to filament-like structures in addition to curled leaves and reduced plant size which was similar to *AG* overexpression in other species, such as *CaMADS1* from hazelnut (Rigola et al. 2001), *Vvmads1* from grapevine (Boss et al. 2001), *NAG1* from tobacco (Kempin et al. 1993), *SAG1* from black spruce (Rutledge et al. 1998), and *LLAG1* from lily (Benedito et al. 2004). The phenotypic alterations of all of these species were similar to those seen after ectopic expression of *AG* in *Arabidopsis* (Mizukami and Ma 1992). Although ectopic expression cannot provide a definitive evaluation of functional homology, it does show that MADS-box proteins from divergent species can maintain some level of functional activity when expressed in the heterologous species (Rutledge et al. 1998).

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## References

- Angenent GC, Franken J, Busscher M, van Dijken A, van Went JL, Dons HJM, van Tunen AJ (1995) A novel class of MADS box genes is involved in ovule development in *Petunia*. *Plant Cell* 7:1569–1582
- Benedito V, Visser PB, van Tuyl JM, Angenent GC, de Vries SC, Krens FA (2004) Ectopic expression of *LLAG1*, an *AGAMOUS* homologue from lily (*Lilium longiflorum* Thunb.) causes floral homeotic modifications in *Arabidopsis*. *J Exp Bot* 55:1391–1399
- Boss PK, Vivier M, Matsumoto S, Dry IB, Thomas MR (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
- Bowman JL, Smyth DR, Meyerowitz EM (1989) Genes directing flower development in *Arabidopsis*. *Plant Cell* 1:37–52
- Brunner AM, Rottmann WH, Sheppard LA, Krutovskii K, DiFazio SP, Leonardi S, Strauss SH (2000) Structure and expression of

- duplicate *AMAGOUS* orthologues in poplar. *Plant Mol Biol* 44:619–634
- Brunner AM, Li JY, DiFazio SP, Shevchenko O, Montgomery BE, Mohamed RM, Wei H, Ma C, Elias AA, VanWormer K, Strauss SH (2007) Genetic containment of forest plantations. *Tree Genet Genomes* 3:75–100
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743
- Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. *Nature* 353:31–37
- Du N, Pijut PM (2009) *Agrobacterium*-mediated transformation of *Fraxinus pennsylvanica* hypocotyls and plant regeneration. *Plant Cell Rep* 28:915–923
- Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G (1997) A polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. *Nature* 386:44–51
- Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res* 16:9877
- Huang H, Mizukami Y, Hu Y, Ma H (1993) Isolation and characterization of binding sequences for the product of the *Arabidopsis* and floral homeotic gene *AGAMOUS*. *Nucleic Acids Res* 21:4769–4776
- Kang HG, Noh YS, Chuang YY, Costa M, An K, An G (1995) Phenotypic alterations of petals and sepals by ectopic expression of a rice MADS box gene in tobacco. *Plant Mol Biol* 29:1–10
- Kempin SA, Mandel MA, Yanofsky MF (1993) Ectopic expression of the tobacco floral homeotic gene *NAG1* converts perianth into reproductive organs. *Plant Physiol* 103:1041–1046
- Kolosova N, Miller B, Ralph S, Ellis BE, Douglas C, Ritland K, Bohlmann J (2004) Isolation of high-quality RNA from gymnosperm and angiosperm trees. *Biotechniques* 36:821–824
- Kramer EM, Alejandra-Jaramillo M, DiStilio VS (2004) Pattern of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. *Genet* 166:1011–1023
- Lefort F, Douglas GC (1999) An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species *Acer*, *Fraxinus*, *Prunus* and *Quercus*. *Ann Des Sci For* 56:259–263
- Li QZ, Li XG, Sun JQ, Zhang XS (2000) Isolation and expression of an *AGAMOUS* homolog in the flower of cucumber (*Cucumis sativus* L.). *Dev Reprod Biol* 9:69–76
- Li QZ, Li XG, Bai SN, Lu WL, Zhang XS (2002) Isolation of *HAG1* and its regulation by plant hormones during in vitro floral organogenesis in *Hyacinthus orientalis* L. *Planta* 215:533–540
- Liu JY, Huang YH, Ding B, Tauer CG (1999) cDNA cloning and expression of a sweetgum gene that shows homology with *Arabidopsis AGAMOUS*. *Plant Sci* 142:73–82
- Ma H, Yanofsky MF, Meyerowitz EM (1991) *AGLI-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev* 5:484–495
- Mizukami Y, Ma H (1992) Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic *Arabidopsis* plants alters floral organ identity. *Cell* 71:119–131
- Mizukami Y, Huang H, Tudor M, Hu Y, Ma H (1996) Functional domains of the floral regulator *AGAMOUS*: characterization of the DNA binding domain and analysis of dominant negative mutations. *Plant Cell* 8:831–845
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Pnueli L, Hareven D, Rounsley SD, Yanofsky MF, Lifschitz E (1994) Isolation of the tomato *AGAMOUS* gene *TAG1* and analysis of its homeotic role in transgenic plants. *Plant Cell* 6:163–173
- Pollock R, Treisman R (1991) Human SRF-related proteins: DNA binding properties and potential regulatory targets. *Genes Dev* 5:2327–2341
- Remphrey WR (1989) Shoot ontogeny in *Fraxinus pennsylvanica* (green ash). II. Development of the inflorescence. *Can J Bot* 67:1966–1978
- Riechmann JL, Meyerowitz EM (1997) MADS domain proteins in plant development. *Biol Chem* 378:1079–1101
- Rigola D, Pe ME, Mizzi L, Ciampolini F, Sari-Gorla M (2001) *CaMAD1*, an *AGAMOUS* homologue from hazelnut, produces floral homeotic conversion when expressed in *Arabidopsis*. *Sex Plant Reprod* 13:185–191
- Rutledge R, Regan S, Nicolas O, Fobert P, Côté C, Bosnich W, Kauffeldt C, Sunohara G, Séguin A, Stewart D (1998) Characterization of an *AGAMOUS* homologue from the conifer black spruce (*Picea mariana*) that produces floral homeotic conversions when expressed in *Arabidopsis*. *Plant J* 15:625–634
- Shore P, Sharrocks AD (1995) The MADS-box family of transcription factors. *Eur J Biochem* 229:1–13
- Skinner JS, Meilan R, Brunner AM, Strauss SH (2000) Options for genetic engineering of floral sterility in forest trees. In: Jain SM, Minocha SC (eds) *Molecular biology of woody plants*. Kluwer Academic, Dordrecht, pp 135–153
- Strauss SH, Rottmann WH, Brunner AM, Sheppard LA (1995) Genetic engineering of reproductive sterility in forest trees. *Mol Breed* 1:5–26
- Theiben G (2001) Development of floral organ identity: stories from the MADS house. *Curr Opin Plant Biol* 4:75–85
- Theissen G, Becker A, Di Rosa A, Kanno A, Kim JT, Munster T, Winter KU, Saedler H (2000) A short history of MADS-box genes in plants. *Plant Mol Biol* 42:115–149
- Tsushima S, van der Krol AR, Chua NH (1993) Ectopic expression of *pMADS3* in transgenic petunia phenocopies the petunia blind mutant. *Plant Cell* 5:843–853
- Wallander E (2008) Systematics of *Fraxinus* (Oleaceae) and evolution of dioecy. *Plant Syst Evol* 273:25–49
- Yanofsky MF (1995) Floral meristems to floral organs: genes controlling early events in *Arabidopsis* flower development. *Annu Rev Plant Physiol Plant Mol Biol* 46:167–188
- Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature* 346:35–39
- Zhang XS, Li QX, Li XG, Bai SN, Lu WL (2000) Hyacinth *HAG1* cloning and expression. *Science in China: C Series* 30:376–381