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

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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 biosafety 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 identify 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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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 MIKC-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)$_n$. 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°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)TCTTG(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 μg total RNA from male inflorescences with dT23VN as primer using Protoscript First-Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA). Using 2 μl out of 20 μl of the cDNA synthesis product, a 20-μl PCR reaction was prepared containing 2.5 μl 10× PCR buffer (Invitrogen, Carlsbad, CA, USA), 1 μl 10 mM dNTP, 0.8 μl 50 mM Mg^{2+}, 1 μl 10 μM AGP1 and AGP2 primers, respectively, and 0.2 μl of 5 U μl^{-1} Taq polymerase. The cycling program consisted of an initial denaturation at 94°C for 2 min, followed by five cycles of 94°C for 30 s, 42°C for 30 s, 72°C for 1 min, 35 additional cycles of 94°C for 30 s, 47°C for 30 s, 72°C for 1 min, and a final extension at 72°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′-TTGAGGAATCTGAGCAGG CTTTC-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′-CCTTCTGTAAG CGCCGCAAC-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 μl of the first-strand cDNA from green ash male inflorescence was used as a template for PCR using AGP7 [5′-GCT CTAGAATGGCATTGCAGA GTGATCAA-3′] (covering the presumed start codon and creating an *Xba*I site) and AGP8 [5′-CCCGAGCTCTCAGA CTAATTGAAGAGGTGG-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 μl out of 20 μl of the cDNA synthesis product was used in a 20-μl 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 μl 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 μg) from leaves was digested with *Eco*RI, *Hin*dIII, 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 [α-^{32}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× SSC containing 0.9 M NaCl and 0.09 M sodium citrate, 5× Dehart's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 mg ml^{-1} denatured salmon sperm DNA. The membrane was then washed at 65°C twice for 20 min in 2× SSC, 0.1% SDS, and once for 10 min in 0.5× 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_1 seeds were placed onto agar plates containing half-strength Murashige and Skoog medium (1962) with 50 mg l^{-1} 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 μmol m^{-2}s^{-1}). Kanamycin-resistant T_1 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

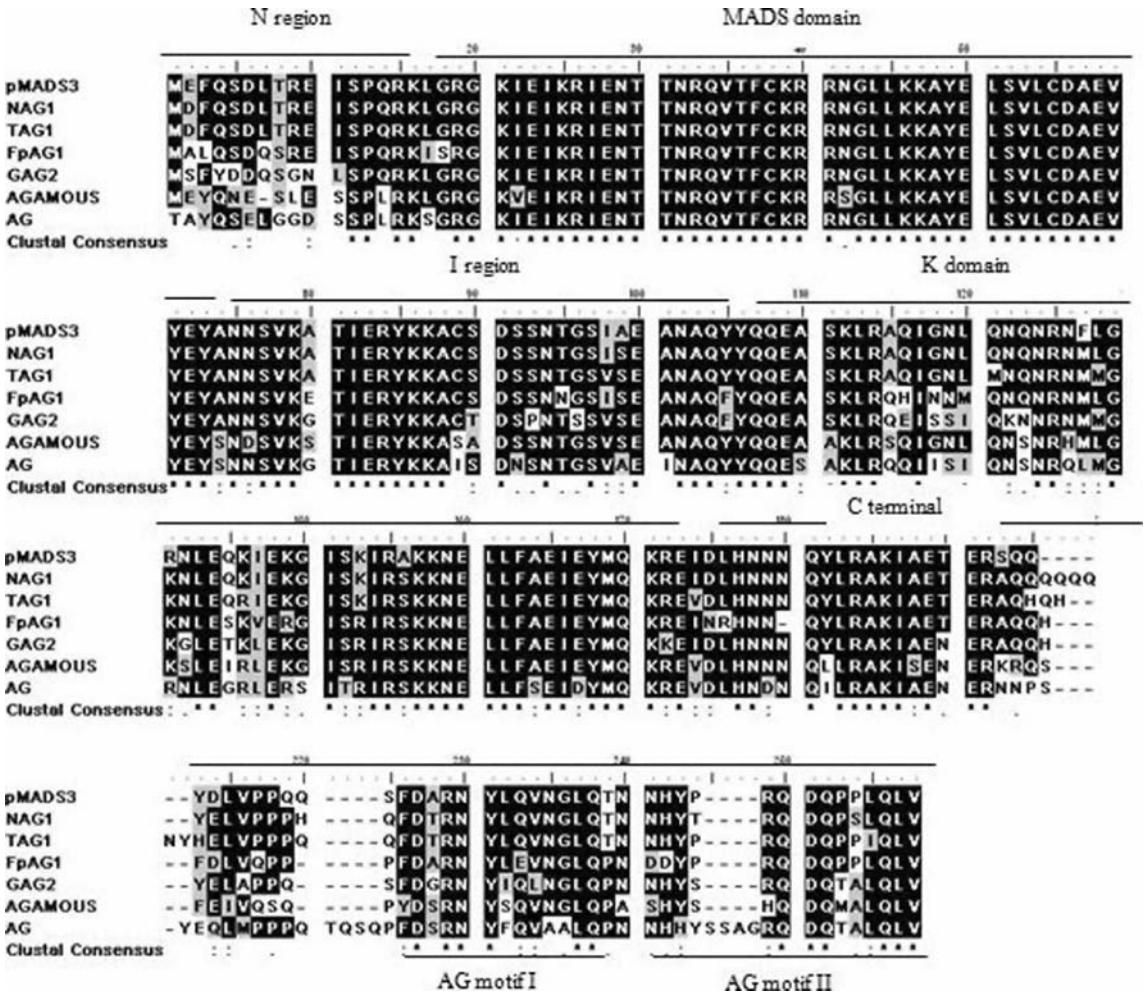

Fig. 1 Alignment of deduced amino acid sequences encoded by *FpAG* with AG homologs from *A. thaliana* (*AG*), *N. tabacum* (*NAG1*), *S. lycopersicum* (*TAGl*), *Petunia* × *hybrida* (*pMADS3*), *P. trichocarpa* (*Agamous*), and *P. ginseng* (*GAG2*). The alignment was generated by the ClustalW program 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_1 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_1 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_1-01, -02, and -03 showed higher expression of *FpAG* with stronger phenotypes than was seen with lines T_1-04, -05, and -06, which had weak phenotypes. The accumulation levels of *FpAG* transcripts in different lines are consistent with phenotypic alterations.

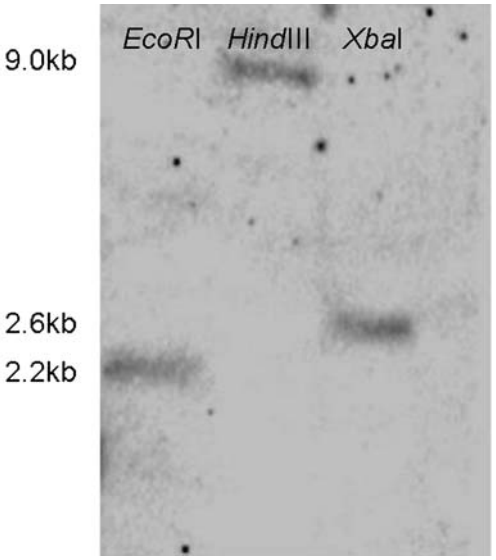

Fig. 3 Southern blot analysis of *FpAG*.The genomic DNA extracted from green ash was digested with *Eco*RI, *Hin*dIII, or *Xba*I. Each *lane* contains 20 μg DNA

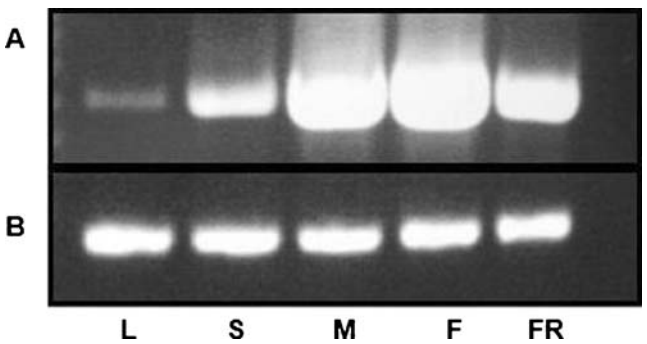

Fig. 2 RT-PCR products from green ash RNA. **A** Products from *FpAG*-specific primers. **B** Products produced with 18S primers, used as a control. *L* leaf, *S* in vitro-germinated seedling, *M* male flower, *F* female flower, *FR* fruit

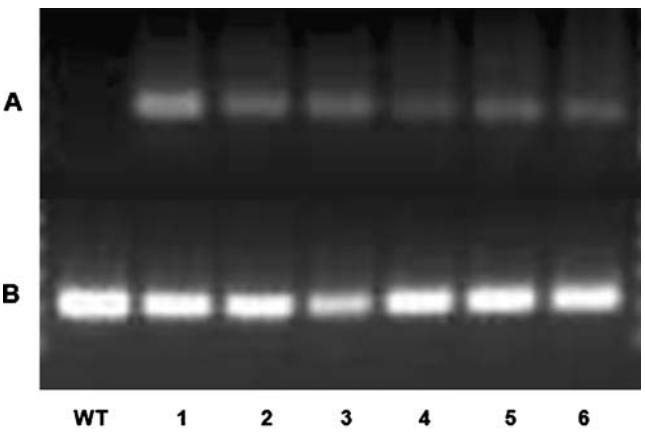

Fig. 4 Overexpression of *FpAG* in *Arabidopsis*. **A** Products of RT-PCR with primers specific for 3′ ends of *FpAG*. **B** Products amplified with 18S-specific primers, used as control (*WT* wild type; *lanes 1–6* individual transgenic lines: T_1-01, T_1-02, T_1-03, T_1-04, T_1-05, and T_1-06)

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-

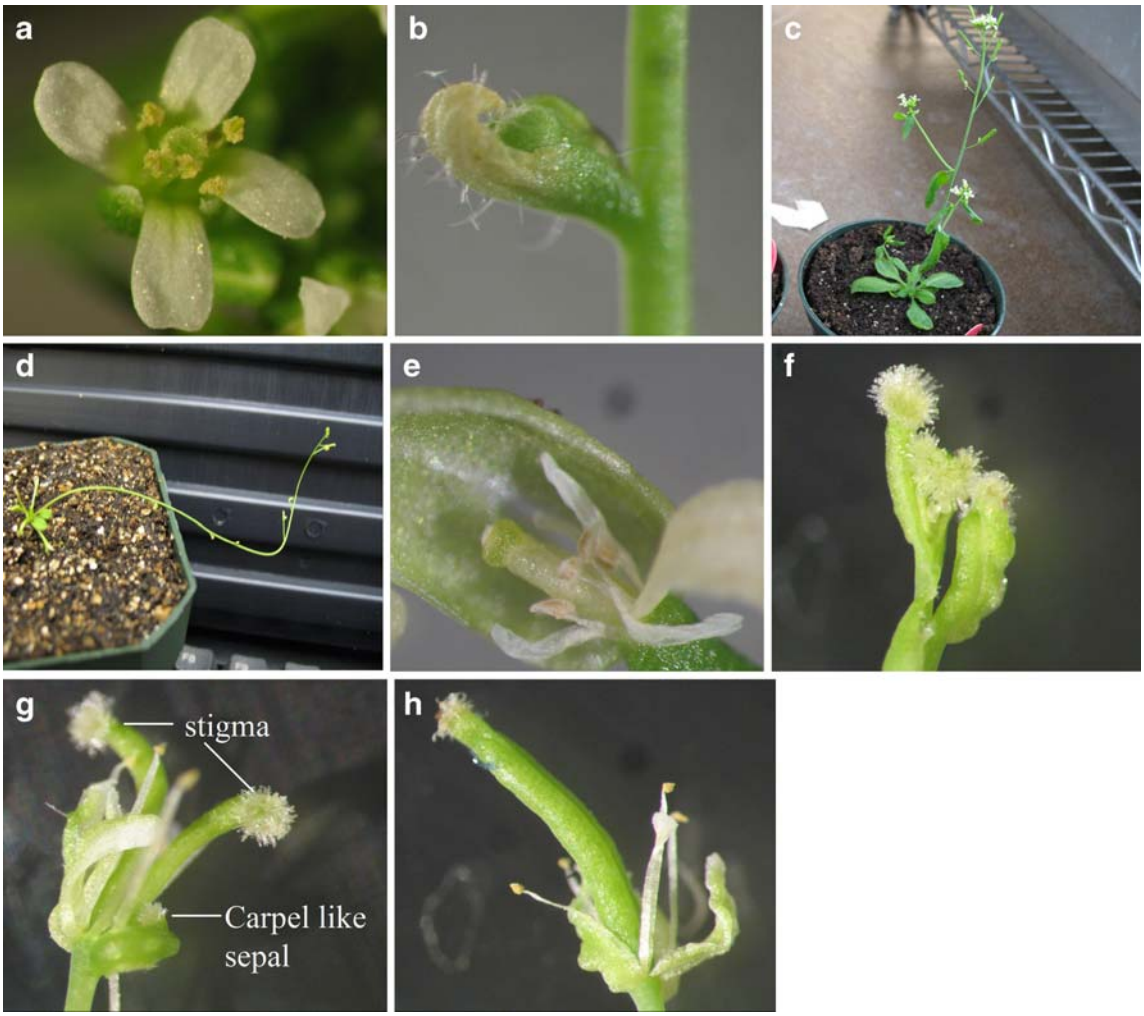

Fig. 5 Floral and vegetative morphology of *Arabidopsis*. **a** Wild-type flower with four sepals, four petals, six stamens, and pistil. **b** Transgenic lateral inflorescence entrapped in curled cauline leaf. **c** Rosette leaves, flower, and seed set of a wild-type plant. **d** Rosette leaves of a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