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Isolation and characterization of a floral homeotic gene in *Fraxinus nigra* causing earlier flowering and homeotic alterations in transgenic *Arabidopsis*

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ABSTRACT

Reproductive sterility, which can be obtained by manipulating floral organ identity genes, is an important tool for gene containment of genetically engineered trees. In *Arabidopsis*, *AGAMOUS* (*AG*) is the only C-class gene responsible for both floral meristem determinacy and floral organ identity, and its mutations produce sterility. As a first step in an effort to develop transgenic sterile black ash (*Fraxinus nigra*), an *AG* ortholog in black ash (*FnAG*) was isolated using reverse transcription polymerase chain reaction and rapid amplification of cDNA ends. Analysis of the deduced amino acid sequence showed a typical MIKC structure of type II plant MADS-box protein with a highly conserved MADS-domain. Phylogenetic analysis revealed that *FnAG* had a close relationship with *AG* orthologs from other woody species. *FnAG* transcript was mainly expressed in reproductive tissues, but rarely detected in the vegetative tissues, consistent with the ABC model for floral development. A functional analysis was performed by ectopic expression of *FnAG* driven by the CaMV 35S promoter in transgenic *Arabidopsis*. Transformed plants showed homeotic conversions of carpeloid sepals and stamenoid petals. Curled leaves, reduced plant size, and earlier flowering were also observed in transgenic *Arabidopsis*. These data indicated that the *FnAG* functions in the same way as *AG* in *Arabidopsis*. These results provide the framework for targeted genome editing of black ash, an ecologically and economically important wetland species.

1. Introduction

Black ash (*Fraxinus nigra* Marsh.) is an economically and ecologically important hardwood species in northeastern North America. The wood is used for cabinets, paneling, flooring, and is preferred by Native Americans for making splints for basketry (Benedict, 2001; Beasley and Pijut, 2013). Black ash also provides food and habitat for wildlife (Leopold et al., 1998) and this species has a great ecological impact, especially in riparian ecosystems (Nisbet et al., 2015). Black ash flowers are perfect or dioecious; they occur in panicles that arise from leaf scar axils produced the previous year (Gucker, 2005). The flowers appear before the leaves.

Emerald ash borer (EAB; *Agrilus planipennis*), an invasive wood-boring beetle from Asia, threatens all North American ash species including black ash with devastating economic and ecological impacts (Poland and McCullough, 2006; Kovacs et al., 2011). In order to manage this aggressive pest and conserve *Fraxinus* spp., there have been numerous calls for genetically engineered ash trees resistant to the EAB. Concerns about transgene flow and its potential impact on the environment, however, limit the widespread acceptance and regulatory approval of transgenic trees (van Frankenhuyzen and Beardmore, 2004). Reproductive sterility, obtained by disrupting flower development, is one of several efficient strategies for gene containment in transgenic crops and trees (Daniell, 2002; Brunner et al., 2007).

Previous studies in model plants established the well-known ABC model to describe the genetic mechanism regulating flower development (Schwarz-Sommer et al., 1990; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991). This model proposed that three classes of homeotic genes act in combination to control floral organ identity: A-class alone controls the formation of sepals; A- and B-classes trigger petal development; B- and C-classes regulate the formation of stamens; and C-class alone directs the formation of carpels. The ABC model has been extended by adding D-class for ovule development (Angenent et al., 1995) and E-class which was required for petal, stamen, and carpel development (Pelaz et al., 2000; Honma and Goto, 2001). According to the ABCDE model, MADS-box proteins interact with DNA to form multimeric complexes that regulate the development of different floral organs (Honma and Goto, 2001).

MADS-box genes are a superfamily of transcription factors found in fungi, animals, and plants. They are distinguished as type I and type II

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(Alvarez-Buylla et al., 2000; Gramzow et al., 2010). The type II MADS-box genes in plants encode MIKC-type proteins that consist of four domains: a highly conserved MADS (M) domain for DNA binding; an intervening (I) domain for the selective formation of DNA-binding dimers; a keratin (K) domain for the formation of an amphipathic helix that promotes protein-protein interaction; and the most variable region, the C-terminal (C) domain, the function of which is not yet known (Theissen et al., 2000). There are around 100 MADS-box genes in flowering plants, but *AGAMOUS* (*AG*) is the only C-class gene found in *Arabidopsis*. A flower of the *Arabidopsis ag* mutant shows petals and new flowers instead of stamens and carpels, respectively, while overexpression of *AG* induces homeotic changes of sepals to carpels, and petals to stamen (*apetala2* (*ap2*)-like phenotype) indicating that *AG* was involved in both floral meristem determinacy and floral organ identity (Yanofsky et al., 1990; Mizukami and Ma, 1992). *AG* homologs have been isolated and studied in a variety of species including woody plants, such as poplar (Brunner et al., 2000), black cherry (Liu et al., 2010), green ash (Du and Pijut, 2010), radiata pine (Liu, 2012), London plane tree (Zhang et al., 2013), and pecan (Zhang et al., 2016). Ectopic expression of *AG* homologs in transgenic plants resulted in homeotic conversion of sepals and petals into carpels and stamens, respectively, which confirmed their function as a C-class floral organ identity gene (Benedito et al., 2004; Du and Pijut, 2010; Wang et al., 2012; Liu et al., 2013; Zhang et al., 2016). *AG* homologs from black ash have not been described.

In the present study, an *AG* ortholog from black ash (*FnAG*) was isolated and characterized as a first step to achieve gene containment in transgenic black ash. Functional homology to *AG* was tested by ectopic expression of *FnAG* in *Arabidopsis thaliana* (with *ap2*-like phenotype in the two outer floral whorls).

2. Materials and methods

2.1. Plant materials

Flowers and leaves were collected in April 2014 from mature male and female *Fraxinus nigra* trees at the Purdue Wildlife Area, Purdue University, and the Ross Hills Park, West Lafayette, IN, USA. Leaves and stems from in vitro shoot cultures of black ash maintained as described by Beasley and Pijut (2013) were also collected for RNA extraction. Samples were immediately frozen in liquid nitrogen and stored at − 80 °C until used for analysis.

Arabidopsis thaliana Col-0 ecotype seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University, Columbus, OH, USA. Seeds were stratified for 3 days at 4 °C in the dark to break seed dormancy, and then sown onto Murashige and Skoog (1962) (MS; M499, PhytoTechnology Laboratories, Shawnee Mission, KS) medium supplemented with 10 g L^{-1} sucrose, 0.5 g L^{-1} 2-morpholinoethanesulfonic acid (MES), 8 g L^{-1} Bacto agar, pH 5.7 in 150 × 15 mm petri dishes. Seeds germinated on agar medium were incubated at 25 °C under a 16 h photoperiod (~100 μmol m^{-2} s^{-1}) provided by cool-white fluorescent bulbs. The germinated seedlings were grown for 2 weeks, and then transferred to water-saturated soil in pots covered with a plastic film to maintain high humidity and placed in the greenhouse under long-day conditions. The plastic film was removed after 2 days.

2.2. Isolation of an AG ortholog from black ash

Total RNA was extracted from the leaves of in vitro shoot cultures using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Re-suspended RNA was treated with DNase I (Thermo Fisher Scientific, Grand Island, NY, USA) in order to remove genomic DNA, and the first-strand cDNA was synthesized from 1 μg total RNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and an oligo-dT primer. Degenerate primers AGP1 and AGP2 (Table 1) were designed based on other *AG* nucleotide sequences to amplify the internal fragment spanning part of the MADS-domain and the K-domain (Du and Pijut, 2010). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with a 25 μl PCR mixture containing 2.5 μl 10 × PCR buffer (5 PRIME, Gaithersburg, MD, USA), 1 μl 10 mM dNTP, 1 μl 10 μM AGP1 and AGP2 primers, respectively, 2 μl cDNA, and 0.25 μl 5 U $μl^{-1}$ Taq polymerase (5 PRIME). 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. A single strong band of expected size (258-bp) was purified with QIAquick Gel Extraction Kit (Qiagen), and was then cloned into pGEM-T Easy vector (Promega, Fitchburg, WI, USA) for sequencing at the Purdue University Genomic Center (West Lafayette, IN, USA). Based on the partial internal sequence of *FnAG*, two sets of gene specific primers were designed to perform 5′- and 3′-rapid amplification of cDNA ends (RACE) (First-Choice RLM-RACE; Life Technologies, Grand Island, NY, USA). For the first-round PCR, AGP3 and AGP4 (Table 1) were used as 5′- and 3′-RACE outer primers, respectively. For the second-round PCR, AGP5 and AGP6 (Table 1) were used as 5′- and 3′-RACE inner primers, respectively. The obtained fragments from the second round PCR were cloned into pGEM-T Easy vectors for sequencing, and then assembled to determine the full-length cDNA sequence. To amplify a complete coding sequence (CDS) and genomic sequence of *FnAG*, the first-strand cDNA and genomic DNA were used as a template, respectively, for PCR using AGP7 and AGP8 (Table 1) as forward and reverse primers, respectively. Unless noted otherwise, all PCR reactions were performed using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions for PCR mixture preparations, and all the cycling programs consisted of an initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The gene structure was determined by aligning the CDS and genomic DNA sequence of *FnAG*.

Table 1
Primer sequences.

Primer	Sequence (5′ - 3′)
AGP1	GGA TCG ARA ACA CVA CAN AYC G
AGP2	GYY TCT TGY TGG TAR WAC TG
AGP3	TGA GGA ATC TGA GCA GGC TTT C
AGP4	CGT CAG GTC ACT TTC TGT AAG C
AGP5	GAC ACT GTC GTT GGC ATA TTC A
AGP6	CTT GCT CAA GAA GGC CTA TGA A
AGP7	ATG GCA TTG CAG AGT GAT CA
AGP8	TCA GAC TAA TTG AAG AGG TGG C
AtAG1	AGG CAA TTG ATG GGT GAG AC
AtAG2	TGG ATC GGA TTC GGG TAA TA
AtActin1	GTC GTA CAA CCG GTA TTG TGC TG
AtActin2	CCT CTC TCT GTA AGG ATC TTC ATG AG

2.3. Phylogenetic analyses

The deduced amino acid sequence of *FnAG* was used to search for *AG* homologs from other plants by BLASTX, and then all sequences were aligned by ClustalW (Larkin et al., 2007). A phylogenetic tree was constructed using the neighbor-joining method in MEGA5 software (Tamura et al., 2011). Bootstrap values were derived from 1000 replicate runs. GenBank accession numbers of amino acid sequences used were as follows: GAG2 (Q40872; *Panax ginseng*), CaMADS (ADU56831; *Coffea arabica*), NAG1 (Q43585; *Nicotiana tabacum*), NbAG (AFK13159; *N. benthamiana*), CaAGL2 (ADP06386; *Capsicum annuum*), pMADS3 (Q40885; *Petunia hybrida*), SiAG (AIS82595; *Sesamum indicum*), CsAG (ADP02394; *Citrus sinensis*), FpAG (AFP99884; *F. pennsylvanica*), VvAG (NP_001268097; *Vitis vinifera*), *AGAMOUS* (× 53579; *A.*