DEVELOPMENT OF TRANSGENIC BLACK ASH (*FRAXINUS NIGRA*) FOR RESISTANCE TO THE EMERALD ASH BORER, AND GENOME EDITING FOR REPRODUCTIVE STERILITY

by

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ABSTRACT

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Black ash (*Fraxinus nigra* Marsh.) is valued for commercial hardwood products such as cabinets, paneling, flooring, and veneer, and for food and habitat for wildlife, specifically in riparian areas. The wood is preferred by Native Americans for making splints for basketry. The emerald ash borer (EAB), an aggressive exotic wood-boring beetle from Asia, is threatening all North American ash species. Since the first EAB infestation in the United States was confirmed near Detroit, Michigan in 2002, it has spread rapidly. In order to manage the EAB and conserve *Fraxinus* spp., there is a need to develop ash trees with resistance to the EAB. The goal of this research was to optimize the genetic transformation and shoot regeneration system for black ash hypocotyls, develop transgenic black ash for EAB resistance and reproductive sterility, and develop an adventitious shoot regeneration protocol using black ash leaves.

An in vitro system for plant regeneration from leaf explants of black ash was successfully developed. Leaf explants were transversally cut across the midrib and cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of plant growth regulators. MS medium supplemented with 22.2 µM 6-benzylaminopurine combined with 31.8 µM thidiazuron produced callus formation (100%) and adventitious shoot bud induction (28.8%). The frequency of regeneration response was significantly higher with compound leaves than single leaflets. Whole plants were acclimatized to the greenhouse.

An *Agrobacterium*-mediated transformation system for black ash hypocotyls was optimized based upon a previously developed protocol in our lab. Sonication for 90 s followed by vacuum-infiltration for 10 min in suspension of *Agrobacterium* strain EHA105 (concentration at OD_{600} = 1.0) was found to be optimal. Silwet L-77 did not make a significant difference on transformation efficiency, but it had a negative effect at high concentration. Using this optimal transformation condition, three independent transgenic lines expressing *Bacillus thuringiensis* (*Bt*) *Cry8D2* gene were obtained. The integration of the full length, intact *Cry8D2* gene was confirmed

by polymerase chain reaction (PCR), and its expression in mRNA and protein level was detected by qPCR and Western blot analysis, respectively. All three transgenic lines contained two copies of the transgene.

An *AGAMOUS* (*AG*) homolog of black ash (*FnAG*) was isolated and characterized as a potential target gene for achieving transgene containment. A 729-bp coding region of *FnAG* was obtained by reverse transcription PCR and rapid amplification of cDNA ends. Deduced amino acid sequence showed a highly conserved MADS-domain. Phylogenetic analysis confirmed that *FnAG* belongs to the clade of C-lineage *AG* subfamily genes. Expression of *FnAG* transcript was detected in the reproductive tissues (female and male flowers), but rarely detected in the vegetative tissues (leaves). Transgenic *Arabidopsis thaliana* plants overexpressing *FnAG* showed *ap2*-like phenotypic alteration and early flowering, indicating *FnAG* functions in the same way as *AG*.

To achieve transgene containment, reproductive sterility can be produced by disrupting genes involved in development of floral organs such as *AG*. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (Cas9) was used to induce mutations at three target sites within *FnAG*. A total of 50 transgenic lines harboring *Cas9* expression cassette were obtained. Of these, only two showed one nucleotide substitution in the target site. *Cas9* transgene silencing in some transgenic lines with no mutations resulted in the low mutagenesis rate.

CHAPTER 1. LITERATURE REVIEW

1.1 Black ash

Black ash (*Fraxinus nigra* Marsh.) is a hardwood tree species native to northeastern North America and eastern Canada. Among 43 species in the genus *Fraxinus* L. (Oleaceae: the olive family), black ash is classified in the section *Fraxinus*, unlike other North American native ash trees such as white ash (*F. americana* L.) and green ash (*F. pennsylvanica* Marsh.), that are grouped in the *Melioides* section. Black ash is closely related to European ash (*F. excelsior*) and Manchurian ash (*F. mandschurica* Rupr.) genetically (based on ribosomal internal transcribed spacer (ITS) data and chloroplast *trn*L-F and *rps*16 data) and morphologically (having a samara with a flattened seed cavity and foliar terminal bud scales) (Wallander 2008). A study of the phylogeny and biogeographic history of ash species demonstrated that early North American ash progenitors in the *Melioides* section spread to Eurasia. The species of the section *Fraxinus* evolved, including Manchurian and European ash. Later, migration of a lineage back into North America led to the evolution of *F. nigra* (Hinsinger et al. 2013). This explains the phylogenetical divergence of black ash and the other North American ash species which are sympatric. Native black ash is typically 12-18 m tall, up to 21 m, forming a single trunk with ascending branches (Anderson and Nesom 2003). The slender trunk is rarely more than 0.6 m in diameter and branches do not appear until high up on the trunk (Collingwood and Brush 1964). The bark is grey and corky, becoming scaly and shallowly furrowed with age. Leaves are deciduous, opposite, and pinnately compound with 7-11 leaflets. Leaflets are 8-14 cm in length and 2.5-5 cm wide, stalkless except only the end leaflet, and they have a finely toothed margin.

Most *Fraxinus* species are dioecious, black ash however is sometimes monoecious, and some trees less commonly produce bisexual flowers (Anderson and Nesom 2003). Small, purplish, usually clustered flowers with a small and deciduous calyx, occur in panicles that arise from leaf scar axils before the leaves (Gucker 2005; Wallander 2008). Flowers are wind-pollinated and produce a single, winged seed. Seed production rarely begins before age 30 years (Heinselman 1981). Black ash seeds with immature embryos need to go through a multistage process of stratification to break morpho-physiological dormancy. The process takes 2-3 years under natural conditions (Benedict and David 2003; Wright and Rauscher 1990).

Black ash most commonly grows in moist to wet soil, especially in swamps, with a native range in wetland forests from Newfoundland west to Manitoba and eastern North Dakota; south to Iowa; east to southern Indiana and West Virginia; and north to Delaware and New Jersey. Black ash typically occurs in poorly drained areas and is tolerant to semi-stagnant conditions, but best growth is on sites with moving, aerated water even though saturated (Wright and Rauscher 1990). Black ash is shade intolerant and prone to windthrow, as it is shallowly rooted. Sprouts can be vigorously produced from root crown or stumps following loss of the stem to wildfire, browsing, or cutting (Anderson and Nesom 2003).

Although black ash wood is not particularly strong, it is used commercially in making cabinets, veneer, paneling, and indoor furniture (Benedict 2001; Gucker 2005). The wood is strongly ring-porous and a dense area of late-wood can be permanently bent, making it ideal for basketry (Farrar 1995; Wright and Rauscher 1990). Native Americans prefer black ash, historically and currently, when making splints for basketry. This practice is most common in Maine, New Brunswick, Nova Scotia, and New York (Gucker 2005). Black ash is also important ecologically, providing a food source and habitat for wildlife. The seed is consumed by game birds, songbirds, and small animals such as squirrels and meadow voles (Leopold et al. 1998). Beaver feed on the bark and wood of black ash; white-tailed deer and moose browse the twigs and foliage (Wright and Rauscher 1990). Black ash provides habitat for amphibians such as frog species in forest wetlands (Marshall and Buell 1955). As black ash is a dominant species in riparian forest areas where it constitutes the interface or transition zone between terrestrial and aquatic ecosystems, it has significant ecological impacts on both ecosystems, playing a role in nutrient and water cycling (Crocker et al. 2006; Nisbet et al. 2015).

1.2 Emerald ash borer

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is an invasive insect pest native to Asia (China, Japan, Korea, Mongolia, and eastern Russia). It is highly destructive of North American native ash trees. While dendrochronological evidence indicated that EAB was accidently introduced into North America during the early 1990s (Siegert et al. 2014), the first EAB infestation in the United States was confirmed near Detroit, Michigan in 2002. Since then, it has spread rapidly through natural dispersal and human-assisted firewood movement, killing tens of millions of ash trees (Poland et al. 2015). By 2009, in some infested

forests in southeastern Michigan, the mortality of black, green, and white ash exceeded 99%, causing a sharp decline in ash density (Klooster et al. 2014). As of September 2017, EAB infestations have been detected in 31 states and two Canadian provinces (http://www.emeraldashborer.info), and it continues to spread each year threatening potentially more than 8 billion ash trees (Mercader et al. 2009).

The economic impacts of the loss of ash trees as a result of EAB, as well as regulations associated with EAB quarantine, are enormous. Black, white, and green ash occupy 7% of all hardwood species in the northeastern United States and eastern Canada, and the value of ash saw timber in the United States represents 7.5% of annual harvests (Granger et al. 2017; USDA-APHIS 2015). In addition to the economic loss to the timber industry, extensive negative economic effects are predicted, as ash is one of the most popular street trees in urban and residential areas. Declining and standing dead trees are hazardous, having the potential to injure people or damage property, so these need to be removed. The potential costs associated with removal of urban ash trees in the United States were estimated at $20-60 billion by Cappaert et al. (2005), although Kovacs et al. (2010) predicted that it will cost $10.7 billion for treating, removing, or replacing EAB-infested ash trees in urban areas.

While it is difficult to quantify, widespread ash mortality could have devastating impacts ecologically, resulting in dramatic shifts in ecosystem structure and function (Ellison et al. 2005). At least 43 native arthropod species that rely exclusively on ash trees for feeding and breeding are at risk of co-extirpation, having potential to lead to cascading impacts on affiliated species (Gandhi and Herms 2010a). Predictable indirect effects include alteration of understory vegetation, soil moisture, soil pH, and nutrient cycles; facilitation of the establishment and spread of light-limited invasive plants; and increased coarse woody debris (Gandhi and Herms 2010b; Herms and McCullough 2014). In particular, in wetland forests where black ash is the dominant canopy species, severe black ash mortality could alter the site hydrology by reducing evapotranspiration during the summer growing season. The resulting alteration of the water table is likely to have numerous adverse impacts on the composition of understory communities (Slesak et al. 2014; Telander et al. 2015).

The impending loss of black ash has cultural impacts on several Native American and First Nation tribes in eastern North America, as they use black ash for basketry. These groups consider

basket making to have an important role in preserving cultural values from generation to generation, as well as providing a source of income (Herms and McCullough 2014).

1.2.1 Identification-life cycle-host range

EAB infestations are difficult to identify during the early stages, but distinct visual signs such as D-shaped exit holes, bark splits, epicormic suckering, and canopy dieback can be observed (USDA-APHIS 2015). Adult beetles are typically metallic-emerald green, 8.5-13.5 mm in length and 3.1-3.4 mm wide. EAB completes a 1- or 2-year life cycle depending on local temperatures or the host tree condition: in cooler climates or in vigorous host trees, EAB was observed to require 2 years to complete development (Cappaert et al. 2005). Adult emergence occurs from mid-May throughout the summer, with a peak in late June or early July [approximately 1,000 growing degree days (GDDs)], from D-shaped exit holes (Brown-Rytlewski and Wilson 2004; Poland et al. 2015). After 10-14 days of feeding on ash leaves and causing minor damage to the host tree, sexually mature adults start mating (Rodriguez-Saona et al. 2007). Female beetles generally lay 60-80 eggs in the cracks or crevices of the bark of susceptible ash tree (Bauer et al. 2004). Larvae then bore through the bark and feed on vascular tissue at the phloem-xylem interface, creating serpentine-shaped galleries that disrupt the flow of water and nutrients (Cappaert et al. 2005). Larvae continue growth until fall completing their fourth instar. Then they burrow into the sapwood, and overwinter as prepupae (USDA-APHIS 2015). Pupation begins in mid-April through May (approximately 400-500 GDDs) and takes about 4 weeks, followed by eclosion and emergence (Bauer et al. 2004).

Native North American ash species are highly susceptible to EAB, although limited resistance was reported in blue ash (*F. quadrangulata*), a species showing non-preference by adult EAB for host selection and feeding (Anulewicz et al. 2007; Pureswaran and Poland 2009; Tanis and McCullough 2012). Species indigenous to Europe encountered by the EAB are also susceptible, such as European ash (*F. excelsior*), flowering ash (*F. ornus*), and raywood ash (*F. oxycarpa*) (Herms, 2015; Orlova-Bienkowskaja 2014). Having coevolved with EAB, however, Asian ash species including Manchurian ash (*F. mandshurica*) exhibit resistance, so only dying or stressed trees are attacked (Liu et al. 2007; Wei et al. 2004). In common garden studies, white and green ash showed higher mortality than Manchurian ash (Rebek et al. 2008). To date, white fringetree (*Chionanthus virginicus* L., Oleaceae) is the only non-*Fraxinus* species that can serve as a larval host for EAB (Cipollini 2015).

1.2.2 Potential control and management

In the early stages of its North American invasion, a great deal of effort was made to contain the areas of nascent infestation and eradicate localized, satellite populations of EAB. These control strategies proved unsuccessful as additional EAB infestations were discovered (Cappaert et al. 2005; McCullough 2015). Once the early eradication efforts were abandoned, state and federal agencies set a goal to slow the rate of ash mortality by reducing EAB population growth (Herms and McCullough 2014). An integrated management strategy was developed under the name of SLAM (SLow Ash Mortality), a project that includes applying insecticide to eliminate adult beetles and larvae; girdling ash trees to attract ovipositing EAB females followed by debarking or destroying the trap trees before the larvae complete their development, and harvesting and utilizing ash trees to reduce the amount of food (ash phloem) available for the development of EAB larvae (Herms and McCullough 2014; McCullough et al. 2009).

Various insecticide formulations and application methods have been tested and proven effective to kill EAB (Herms et al. 2014; Poland et al. 2016). While some chemicals need to be applied yearly, emamectin benzoate, in particular, showed consistent results in controlling EAB at least 2 years with a single application (Smitley et al. 2010). Chemicals can be applied systemically through soil injections or drenches. These methods are preferred because of their low impacts on non-target organisms and low applicator exposure, but basal trunk injections or sprays are also available to control EAB (Cappaert et al. 2005; Herms et al. 2009). Insecticidal controls, however, are expensive and have adverse effects such as poisoning of non-target beneficial insects and environmental contamination when applied repeatedly (Kreutzweiser et al. 2007; Ibrahim and Shawer 2014). Insecticidal treatments are not practical on a large scale or in riparian forest areas where black ash is the dominant species. Nevertheless, insecticide options may be able to suppress EAB populations and protect ash trees, particularly in the urban landscape.

Meanwhile, studies have been conducted to investigate the potential of biocontrol using parasites (Belokobylskij et al. 2012; Liu et al. 2003; Yang et al. 2008). Three parasitoid species from China and one species from Russia have been imported and released that include an egg parasitoid, *Oobius agrili*, and three larval parasitoids, *Tetrastichus planipennisi*, *Spathius agrili*, and *Spathius galinae* (USDA–APHIS/ARS/FS 2016). As some of these parasitoid species failed to establish because of cold weather, additional exploration and evaluation will be needed to provide species capable of establishing in the diverse climate zones of North America (Bauer et al.

2015; Herms and McCullough 2014). Researchers have developed methods for monitoring and are continuing to evaluate the parasitoid efficacy reporting some positive results (Abell et al. 2014; Bauer et al. 2015; Parisio et al. 2017).

Development of EAB-resistant ash would be a promising solution to conserve ash trees. Methods for developing EAB-resistant ash include breeding or introducing resistance genes through genetic transformation. As Manchurian ash appeared to be more resistant to EAB than native North American ash species, studies of resistance to EAB have focused on elucidating factors that impart resistance in Manchurian ash. Metabolomic, transcriptomic, and proteomic analyses have been conducted to find differences between resistant- and susceptible-species (Bai et al. 2011; Cipollini et al. 2011; Eyles et al. 2007; Showalter et al. 2015; Whitehill et al. 2012). In addition to the endogenous genes that may confer resistance, natural toxin genes originated from bacteria such as *Bacillus thuringiensis* (*Bt*) could be used to develop transgenic ash trees resistant to EAB.

1.3 *Bacillus thuringiensis* (*Bt*)

Since the first identification and description of its toxic effects in the early twentieth century, *Bt*, a naturally occurring bacterium has been widely used to control plant-eating insects. During sporulation, *Bt* produces insecticidal crystalline proteins (Cry proteins) composed of δ-endotoxin which has a specific toxic effect against certain insect orders such as Lepidoptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga as well as nematodes (Bravo et al. 2017). In order to be effective, Cry proteins need to be converted into the active form by protease under high pH condition (pH>9) in the insect midgut during digestion (Ibrahim and Shawer 2014), and this character makes *Bt* products safe to mammals including humans which have an acidic digestive system. The Cry proteins then bind to specific receptors on the midgut cell membrane, forming pores and leading to leakage, resulting in cell death by osmotic lysis (de Almeida Melo et al. 2016). In addition to this lytic mechanism of toxicity of Cry proteins, a signaling pathway model was also proposed. According to this hypothesis, the Cry toxin connected to its receptors causes alteration in cellular metabolism by activation of ion channels in the plasma membrane that stimulates abnormal movement of ions, resulting in apoptosis (Zhang et al. 2005).

Currently more than 750 different *Bt* genes have been characterized and classified in 74 classes of Cry proteins (Crickmore et al. 2016). Each Cry protein has high affinity and specificity

to their respective receptors only in target insects. Bauer and Lodoño (2011) reported that Cry8Da toxin produced by *Bt* SDS-502 strain was effective to kill adult EAB, showing a narrow spectrum of activity. Although *Bt* toxin can be applied directly to plants as powders or liquid sprays, its commercial use as bio-insecticide is limited because of high costs of production and short duration as a result of rapid degradation by UV or rain wash (Navon 2000). Instead, *Bt* genes encoding Cry proteins can be genetically introduced into plant genomes that allows Cry proteins to be produced within the plant.

1.3.1 Transgenic *Bt* trees

Bt gene transfer through biotechnology has been achieved for numerous plant species and successfully resulted in the production of plants with resistance against major agronomic insect pests. Because most Cry proteins, even within the same protein subfamily, showed distinctive insecticidal effect, different *Bt* genes can be incorporated into a crop to confer resistance against different target insects, and two or more *Bt* genes can be stacked to enhance their efficacy. In addition, as bacterial codon usage which has adenine and thymine-rich regions is not compatible with expression in plants, the coding sequence should be modified and optimized to plant use (Jackson et al. 2014).

Since the first *Bt* transgenic cotton (*Gossypium hirsutum*) was commercialized in 1996, as of 2015, *Bt* crop species approved for field release in the United States, include cotton, corn (*Zea mays*), potato (*Solanum tuberosum*), soybean (*Glycine max*), and tomato (*Solanum lycopersicum*) (National Academies of Sciences, Engineering, and Medicine 2016). The advantages of using *Bt* transgenic plants include: 1) constitutive production of Cry proteins during the entire cultivation period with no need of repeated pesticide application; 2) lower cost with reduced use of broad-spectrum chemical pesticides; and 3) higher farmer profits (Ibrahim and Shawer 2014; Tabashnik et al. 2013). For these reasons, transgenic *Bt* crops have been vigorously adopted worldwide by farmers. There has been a sharp increase of the total area of land cultivated with *Bt* crops: from 1.1 million hectares in 1996 to 66 million hectares in 2011 (Tabashinik et al. 2013). In 2016, *Bt* corn and *Bt* cotton accounted for 79% and 84% of all planted corn and cotton in the United States, respectively (https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx).

Along with advances in transgenic *Bt* crops, a large number of studies have been carried out to develop transgenic trees for insect resistance through overexpression of *Bt* genes. Poplar species, as models for forest tree molecular biology, have been used extensively to study *Bt* transgenic trees since the first *Bt* expressing tree was reported in 1991 (Axelsson et al. 2012; Génissel et al. 2003; Klocko et al. 2014; McCown et al. 1991; Wang et al. 1996; Zhang et al. 2011). Although genetically modified forest trees are not yet allowed for commercial use in most of the country, *Bt* poplars were approved for commercialization and have been planted in plantation forests in China since 2003. By 2016, 543 hectares of *Bt* poplar have been planted. *Bt* poplars showed a substantial improvement in insect resistance, with extremely low probability of transgene flow (Hu et al. 2014, 2017; ISAAA 2016). Various *Bt* transgenic trees have also been successfully developed in other species, including *Pinus* (Grace et al. 2005; Tang and Tian 2003), *Eucalyptus* (Harcourt et al. 2000), *Picea* (Lachance el al. 2007), and *Larix* (Shin et al. 1994).

In addition to using *Bt* genes, a few studies examined upregulation of endogenous resistance genes, such as ascorbate oxidase and MYB134 tannin regulatory gene. Results from these experiments have been mixed; generating adverse results of increasing caterpillar feeding (Boeckler et al. 2014) or no significant effect on the insect (Barbehenn et al. 2008). Introduction of *Bt* genes generally showed high mortality of insects (Hjältén and Axelsson 2015). Transgenic American chestnut trees expressing a wheat oxalate oxidase gene showed resistance to the blight fungus (*Cryphonectria parasitica*) (Newhouse et al. 2014). More studies on endogenous defense traits need to be made. For now, the introduction of a *Bt* gene (*Cry8Da*) could be a feasible method for achieving EAB-resistance in black ash.

1.4 Transgene containment

During the past few decades, transgenic biotechnology has had tremendous positive impacts on modern agriculture, including enhanced crop production which helps increase farm incomes and food security, reduced herbicide and pesticide application which protects the environment, and the potential for solving malnutrition problems in developing countries. As the cultivation area of transgenic crops has expanded rapidly, many concerns and controversies have surfaced, mainly about biosafety issues. The most debated biosafety issue is transgene escape, a process of transgene movement from transgenic plants to wild or weedy relatives through gene flow (Lu and Wang 2012), and its potential environmental impacts. Gene flow can primarily

happen via dispersal in viable pollen, although it possibly occurs via seed, or vegetative propagules. Pollen-mediated gene flow depends on the intrinsic biological features of a species such as the amount of pollen produced, longevity of pollen, and compatibility, and also on environmental conditions, including the distance between transgenic and other pollen recipient plants, and the strength and direction of wind (Daniell 2002; Rong et al. 2010). Seed-mediated gene flow may occur through the natural dispersal by animals or wind, and also through human-influenced seed movement during harvest, transportation, and planting (Lu and Wang 2012). Vegetative propagules, such as rhizomes, roots, and tubers may also mediate gene flow (Mallory-Smith and Zaiola 2008).

Although scientific evidence to date indicates transgenic crops are safe for animal and human consumption and beneficial to the environment (National Academies of Sciences, Engineering, and Medicine 2016), concerns relative to the potential environmental impacts of transgene flow have consistently been raised. The concerns include: the adventitious mixing of transgenic and non-transgenic crops (called "contamination") that may cause trading conflicts between countries or food biosafety concerns, persistence or invasiveness of existing weeds, the possibility that transgene flow could result in weeds with a fitness advantage, and the belief that widespread transgenic crops may cause undesired changes in the genetic diversity of weed, insect, or traditional crop populations (Dale et al. 2002; Lu and Wang 2012; Mallory-Smith and Zapiola 2008). Science-based, long-term examinations are needed to evaluate these concerns.

1.4.1 Regulation of transgenic forest trees

Although transgenic crops have been widely adopted and have brought substantial benefits, transgenic forest trees have not yet been approved to be planted commercially, with two exceptions: *Bt* poplar in China and fast-growing transgenic eucalyptus in Brazil (ISAAA 2016). Stringent regulations and low public-acceptance make deployment of transgenic forest trees lag far behind agricultural transgenic crops. Characteristics of forest trees such as a relatively long life span, part of a wild resource, and major constituent of an ecosystem, unlike other agricultural crops in controlled production systems, make transgenic forest trees scrutinized in political and environmental policies, and as well as a sociocultural issue. The Forest Stewardship Council (FSC), an international certification system of sustainably managed forests, prohibits plantation of all forms of transgenic forest trees (Strauss et al. 2001). In the United States, transgenic forest trees

are regulated by three agencies: 1) United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS), which regulates all transgenic plants including forest trees that could affect agriculture and the environment; 2) Environmental Protection Agency (EPA), which regulates uses of plant-incorporated protectants such as pesticidal properties of transgenic trees; and 3) Food and Drug Administration (FDA), which regulates transgenic trees that produce feed and food (Strauss and Viswanath 2011). Current regulation of transgenic forest trees is based on the process, but not on the product. However, recent developments in biotechnology such as targeted genome editing may blur the line between conventional and transgenic approaches (Araki and Ishii 2015), making the current process-based regulation of transgenic forest trees obsolete and unreasonable. As these regulations are major obstacles to the development and application of transgenic forest trees, reliable and case-by-case based assessment should be adopted.

Transgenic forest trees offer several potential benefits. A primary aim would be to impart pest resistance and improve wood quality that would contribute to enhanced yields and profits for the forest products sector. Biotechnology could also be used for conservation and restoration of endangered species such as ash and American chestnut species threatened by EAB and chestnut blight, respectively. Transgenic trees could also be used to advance fundamental research in tree biology. Field tests of loss- or gain-of-function in transgenic lines would elucidate gene functions or gene regulatory mechanisms.

Since 2009, the Forest Health Initiative (FHI) has proposed possible approaches for using transgenic forest trees to protect trees against emerging threats (http://foresthealthinitiative.org/index.html). The first case study of transgenic American chestnut that resists chestnut blight involved extensive research to test the safety and efficacy of a transgenic forest tree. Meanwhile, coordinated efforts have been made to develop a better understanding of the societal benefits of transgenic forest trees and to address regulatory requirements.

1.4.2 Reproductive sterility

As part of an effort to get regulatory approval, various transgene containment strategies have been suggested. These include reproductive sterility, plastid transformation, temporal- and tissue-specific control, and transgenic mitigation (Daniell 2002). The most extensively studied approach, reproductive sterility, can be achieved through control of the development of

reproductive structures. A cytotoxin gene driven by a floral tissue-specific promoter has been used to interfere with pollen formation, resulting in male sterility. A stilbene synthase gene fused to a male cone-specific promoter from *Pinus radiata* caused ablation of pollen formation in anthers of transgenic tobacco that gave a high rate of male sterility (Höfig et al. 2006). A ribonuclease gene from *Bacillus amyloliquefaciens*, *BARNASE*, has been used under the control of tapetal-specific promoters to create male sterility in many plants including tobacco, oilseed rape, tomato, and tree species as well (Medina et al. 2013; Roque et al. 2007). A complete pollen control phenotype was achieved in transgenic pine and eucalyptus trees, representing gymnosperm and angiosperm trees, using *BARNASE* driven by a pine male cone-specific promoter (Zhang et al. 2012). In a field trial of transgenic hybrid poplar after two growing seasons, stable male-sterility was successfully obtained through the expression of a *BARNASE* gene driven by the TA29 tapetum-dominant promoter, with no visible pollen production in any transgenic trees (Elorriaga et al. 2014). Because many floral predominant promoters are not expressed exclusively in floral tissues, low levels of BARNASE can be expressed in vegetative tissues, causing some side-effects of growth inhibition and morphological alteration (Brunner et al. 2007). Growth retardation and abnormal morphology were observed in BARNASE transgenic poplar and birch (Elorriaga et al. 2014; Lännenpää et al. 2005; Wei et al. 2007). A natural inhibitor of BARNASE, Barstar, has been added under the control of a weak constitutive promoter in order to reduce the side-effects in non-targeted tissue (Kobayashi et al. 2006; Wei et al. 2007). However, the Barstar-mediated attenuation of BARNASE varied with *barstar:barnase* expression ratio and was substantially reduced after one or two growing seasons in the field (Wei et al. 2007). Thus, strict scrutiny should be applied in selection of promoters and cytotoxins for exploiting the ablation-induced male sterility system for tree species.

Instead of floral tissue ablation via a cytotoxin, reproductive sterility can also be obtained by disrupting flowering genes or by interfering with their expression. RNA interference (RNAi) has been used to induce sequence-specific gene suppression of the *LEAFY* gene in poplar, resulting in a decrease in inflorescence size and loss of functional sexual organ development that produced sterility (Klocko et al. 2016b). Suppression of two co-orthologs of *AGAMOUS* in apple by RNAi triggered double flowers that had few visible pollen grains and a decreased number of stigmas, resulting in reduced fertility (Klocko et al. 2016a). Chimeric Repressor gene-Silencing Technology (CRES-T) has been applied to generate male sterility in *Arabidopsis*, rice, and tall fescue by

causing downregulation of *APETALA3*, *AGAMOUS*, and *LEAFY* (Mitsuda et al. 2006; Sato et al. 2012). RNA-mediated silencing has some limitations, however, including non-target effects, variability, and incompleteness of knock-downs (Madhani 2006; Mansoor et al. 2006). To address these limitations, recently emerging targeted genome editing techniques can be used to directly and precisely disrupt the target floral genes to produce knock-out mutations.

1.4.3 *AGAMOUS*

The well-known ABCDE model describes the genetic mechanism regulating flower development. According to this model, homeotic genes in each class function in combination to control the development and specification of floral organs, giving rise to sepals, petals, stamens, and carpels. With the exception of *APETALA2* (*AP2*), most of the genes belonging to the ABCDE model are members of the MADS-box gene family (Theißen et al. 2001). *AGAMOUS* (*AG*) is the only C-class gene found in *Arabidopsis*. It is responsible for stamen and carpel formation in the third and fourth whorls, respectively. In addition, *AG* is involved in floral meristem determinacy such that *ag* mutant flowers produced petals and new flowers, instead of stamens and carpels, resulting in complete sterility (Yanofsky et al. 1990). Ectopic overexpression of *AG* caused homeotic alterations of carpel-like sepals and stamen-like petals (*ap2*-like phenotype) in transgenic *Arabidopsis* (Mizukami and Ma 1992), because C-class genes antagonize A-class genes such as *AP2*.

AG transcript was detected evenly throughout third and fourth whorl organ primordia in the early stage of flower development, and then remained high throughout most of flower development (Bowman et al. 1991; Ryan et al. 2015). The expression of *AG* was restricted in the first whorl by AP2, while it was activated by LEAFY, partly in cooperation with WUSCHEL (Krogan et al. 2012). In *Arabidopsis*, the temporal and spatial expression of *AG* was controlled by a *cis*-element within the second intron of *AG* (Causier et al. 2009). Loss of this regulatory element resulted in a 40% decrease in *AG* gene expression without changing the splicing of the gene (Yan et al. 2016). A microarray expression profiling of wild-type and *ag* mutant flowers showed that more than 1,000 genes were regulated downstream of *AG* (Wellmer et al. 2004). A comprehensive study, combining genome-wide localization studies and gene perturbation experiments, demonstrated that *AG* controls the expression of downstream genes which mediate a plethora of different developmental processes, in conjunction with B-class genes *APETELA3* and

PISTILLATA (*PI*) (ÓMaoiléidigh et al. 2013). These authors found that trichome initiation, an example of the leaf development program, was inhibited by AG in emerging floral primordia. Ng et al. (2009) proposed that AG regulates several key transcriptional regulators that pattern the gynoecium by direct interaction with GIANT KILLER (GIK), a chromatin modifier which serves as a molecular node.

Because of its important role in the formation of the reproductive organs and the control of floral meristem determinacy, *AG* has been considered a target gene for knock-out mutation to produce reproductive sterility in transgenic plants. *AG* orthologs have been extensively isolated and characterized in various plant species, including green ash (Du and Pijut 2010), poplar (*Populus trichocarpa*) (Brunner et al. 2000), black cherry (*Prunus serotina*) (Liu et al. 2010), black spruce (*Picea mariana*) (Rutledge et al. 1998), pine (*Pinus radiata*) (Liu 2012), London plane tree (*Platanus acerifolia*) (Zhang et al. 2013), eucalyptus (*Eucalyptus grandis*) (Kato and Hibino 2009), Japanese apricot (*Prunus mume* Sieb. et Zucc.) (Hou et al. 2011), and pecan (*Carya illinoinensis*) (Zhang et al. 2016). These *AG* orthologs shared a highly conserved MADS-domain. Constitutive expression of *AG* orthologs in transgenic *Arabidopsis* or tobacco resulted in *ap*2-like homeotic alterations that demonstrated the orthologs function in the same way as *AG* in flower development (Du and Pijut 2010; Hou et al. 2011; Rutledge et al. 1998; Zhang et al. 2013; Zhang et al. 2016).

1.5 Targeted genome editing

Genome editing, the ability to manipulate a genome at targeted sites, is a powerful tool for basic research to analyze gene function as well as for genetic improvement by providing precise and predictable means to modify genomes. In the last few decades, huge advances have been made in targeted genome editing techniques with the emergence of engineered nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (Gaj et al. 2013). These systems share a common fundamental principle that relies on the double-stranded breaks (DSBs) generated by engineered nucleases at a target genomic locus. DSBs are then repaired through endogenous DNA damage repair mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR). During the re-ligation process of the DSBs through NHEJ, several base insertion or deletion (indels) are often formed (Lieber 2010). If the indels make premature stop codons or frameshift mutations within a coding exon, the target gene is disrupted.

This error-prone feature of NHEJ can be used to generate knockout mutants which are useful for functional and reverse genetic studies, and for enhancing agricultural traits. When two DSBs are introduced simultaneously, the intervening fragment can be removed or inverted (Cong et al. 2013). Alternatively, in the presence of an exogenously introduced repair template with homology to the target locus, DSB can be repaired through the HDR pathway (Voytas 2013). This homologous recombination (HR) technique via HDR can be exploited to achieve gene correction, gene replacement, and gene stacking at the targeted genomic site (Malzahn et al. 2017).

ZFNs and TALENs are artificial fusion proteins that consist of an engineered DNA-binding domain and the nonspecific nuclease domain of FokI restriction enzyme (Christian et al. 2010; Kim et al. 1996; Miller et al. 2011). Because FokI nuclease functions as a dimer, a pair of ZFNs or TALENs, which recognize the sequence on the opposite strands, is required to cleave double strands. ZF DNA-binding domain from ZF transcription factor consists of approximately 30 amino acids that specifically binds to three successive bases. Each ZFN is typically composed of three to four ZF domains that can recognize 9-12 bp of target DNA sequences. A spacer of 5-7 bp between two ZFN target sites is required to provide a microenvironment for the dimerization of FokI cleavage domain. Although ZFNs were utilized in various organisms including plants, difficulties in designing ZF binding domains have limited their application (Sander et al. 2011; Zhang et al. 2017). Similar to ZFNs, TALENs use the TALE DNA-binding domain, a series of repeat domains composed of 34-35 amino acids that come from the bacterium *Xanthomonas*. Each repeat binds to a specific base via the two amino acids in position 12 and 13 [(called the repeat variable diresidue (RVD)]. While ZF arrays recognize DNA triplets, TALE repeats can recognize only a single base, conferring more specificity and flexibility to design DNA-binding domains than ZFNs. This technique has been widely applied to plant species such as rice (Li et al. 2012), tomato (Lor et al. 2014), maize (Liang et al. 2014), and soybean (Haun et al. 2014). As TALENs still require large modular proteins for DNA-binding, however, their design and construction is difficult, laborious, and expensive (Sander et al. 2011). Most recently, a RNA-dependent DNA-recognition system using a bacterial monomeric endonuclease, known as CRISPR/Cas9 is being rapidly developed. CRISPR/Cas9 is unlike the protein-based DNA-binding systems of ZFNs and TALENs.

1.5.1 CRISPR/Cas9

The CRISPR/Cas9 system is an adaptive immune system of bacteria and archaea against invading foreign DNA (i.e., a virus or plasmid) (Sorek et al. 2013). In the type II CRISPR/Cas9 system from *Streptococcus pyogenes*, which is the most studied and utilized system, the immunity is acquired by three steps: 1) short sequences from invading DNA, known as spacers, are integrated into a CRISPR locus, 2) the CRISPR arrays are transcribed and then processed forming short CRISPR RNAs (crRNAs), which are subsequently activated by combining with the transactivating CRISPR RNA (tracrRNA), and 3) the crRNA-tracrRNA complex directs Cas9 to the target sequence for cleaving the invading DNA (Zhang et al. 2017).

This bacterial defense machinery has been rapidly adopted for use as an RNA-guided Cas9 gene editing tool. Twenty nucleotides of crRNA and tracrRNA can be combined into a chimeric single guide RNA (sgRNA) that can be reprogrammed to target any sequence and co-delivered with a Cas9 expression cassette (Cong et al. 2013; Jinek et al. 2012). To recognize the target sequence, a conserved protospacer-adjacent motif (PAM), usually NGG, is required directly downstream of the target sequence (Jinek et al. 2012). Once Cas9/sgRNA complex binds to the target sequence, two independent nuclease domains in Cas9, HNH and RuvC, cleave three bases upstream of PAM on the complementary strand and the opposite strand, respectively, leaving a blunt end DSB (Nishimasu et al. 2014). Because of its simplicity, high efficiency, and design flexibility, the CRISPR/Cas9 system has been extensively used for genome editing in various organisms, including plants (reviewed in Malzahn et al. 2017).

NHEJ based plant genome editing by CRISPR/Cas9 is the most widespread mechanism to create directed mutations. Depending on the developmental stages when the mutagenesis event occurs, four different types of mutants can be made in a diploid plant: heterozygous, if only one locus is edited; homozygous, if both alleles are edited to have the same mutation; biallelic, if both alleles are edited but NHEJ results in different mutations; and a chimeric plant can be made when the mutagenesis occurs later in development and independently in different tissues (Belhaj et al. 2015). This feature was proven by targeting the *PHYTOENE DESATURASE* (*PDS*) gene, which is involved in chlorophyll biosynthesis, thus mutation in this gene resulted in an albino phenotype in the first generation (Fan et al. 2015; Pan et al. 2016; Zhang et al. 2014). Beyond proof-of-concept, this mechanism has been applied for enhancing desirable traits. Wang et al. (2016) reported enhanced rice blast resistance by knocking out the *OsERF922* gene. Similarly, potyvirus

resistance was developed in *Arabidopsis* by introduction of sequence-specific mutations at eIF(iso)4E locus (Pyott et al. 2016). CRISPR/Cas9-mediated mutagenesis of multiple yield-related genes in rice resulted in enhanced grain number, dense erect panicles, and larger grain size (Li et al. 2016). In maize, male sterility was obtained by knocking out two male fertility genes (Ms26 and Ms45) (Svitashev et al. 2015). As CRISPR/Cas9 allows multiplex gene editing via simultaneous introduction of two or more sgRNAs, this feature can be used to make large chromosomal deletions or to knock out many target genes as well as redundant genes at the same time.

Although HDR based genome editing by CRISPR/Cas9 is far less efficient than NHEJ, if a sufficient amount of repair template as well as Cas9 can be delivered it could be a powerful tool for gene replacement, gene stacking, and tagging in a plant genome (Malzahn et al. 2017). A stable and heritable integration of the kanamycin resistance gene was confirmed at the *ADH1* gene locus in *Arabidopsis* (Schiml et al. 2014). Multiple precise point mutations in the *ALS* gene by CRISPR/Cas9-mediated HDR conferred herbicide resistance in rice and maize (Sun et al. 2016; Svitashev et al. 2015). To increase the efficiency of delivery, some researchers deploy a geminivirus replicon system that can replicate within the plant nucleus and provide a high copy number of the repair template (Butler et al. 2016; Wang et al. 2017b). In tomato, a strong 35S promoter was introduced upstream of *ANT1*, a gene controlling anthocyanin biosynthesis, resulting in high accumulation of anthocyanin that produced a purple plant phenotype (Čermák et al. 2015).

Similar to other genome editing tools, CRISPR/Cas9 has a possible limitation of the potential off-target issue, although the off-target mutation frequencies in plants are quite low. A number of approaches have been studied to minimize the off-target activity. The specificity of CRISPR/Cas9 is primarily determined by 8-12 nt proximal to PAM (named 'seed sequence'), but mismatches in the PAM-distal region can be tolerated, which may cause unwanted mutations at off-target sites (Fu et al. 2013; Hsu et al. 2013). Fu et al. (2014) reported that truncated sgRNAs only 17 and 18 nt in length improved specificity more than 5,000 times without sacrificing on-target editing efficiency. Alternatively, Cas9 nickase mutants, which are generated by mutations in one of the two nuclease domains in Cas9, have been developed to induce cleavage only at one strand. A pair of Cas9 nickases coupled with two distinct sgRNAs targeting adjacent sequences were directed a complete DSB with double nicks, reducing off-target events (Ran et al. 2013; Shen et al. 2014). Another approach has been suggested by fusing a catalytically inactive Cas9 (dCas9)

to the FokI nuclease, named fCas9 (Guilinger et al. 2014; Tsai et al. 2014). A pair of fCas9s that bind to target sites with a spacer of 15 or 25 bp showed higher specificity more than 140-fold and at least four-fold higher compared to wild-type Cas9 and Cas9 nickases, respectively (Guilinger et al. 2014).

1.5.2 Genome editing of woody plants

To date, a limited number of studies of genome editing have been reported in woody plants. The first report of ZFN-mediated genome editing of a woody plant was made by Peer et al. (2015) in two perennial fruit trees, apple and fig. Inactivated *uidA* transgene, which had a premature stop codon within the ZFN target site, was successfully mutated via ZFN, leading to restoration of the gene and GUS expression. In order to avoid toxicity of ZFN, the authors used *Arabidopsis* heat-shock-inducible promoter (Peer et al. 2015). Lu et al. (2016) tested four pairs of ZFNs targeting *LEAFY* (*PtLFY*) and two *AG* orthologs (*PtAG1* and *PtAG2*) in *Populus*, reporting the lowest mutation rate of ZFN mutagenesis in plants. Among over 21,000 transformed explants, only two ZFN transgenic plants were developed with heterozygous 7 bp deletion in the *PtAG2* and no mutations were observed in the *PtAG1* or *PtLFY*, showing 0-0.3% mutation rate per explant per allele (Lu et al. 2016).

Jia and Wang (2014) reported the first application of CRISPR/Cas9 in a woody plant using sweet orange, *Citrus sinensis*. Transient agroinfiltration was facilitated by pre-treatment with *Xanthomonas citri* subsp. *citri*. They generated mutations within the targeted *CsPDS* locus with a 3.2 to 3.9% mutation rate (Jia and Wang 2014). In apple, loss-of-function of the apple *PDS* gene was obtained by CRISPR/Cas9, resulting in albino phenotypes (Nishitani et al. 2016). Notably, an 18 bp truncated gRNA successfully induced a targeted mutation. More recently, citrus canker-resistant grapefruit (*Citrus paradisi*) was developed by induction of mutations in the canker susceptibility gene, *CsLOB1*, using CRISPR/Cas9 (Jia et al. 2017). Among six independent CRISPR lines, no canker symptoms were observed from four lines, proving the efficacy of CRISPR/Cas9-mediated genome editing for breeding disease-resistance.

Populus is the only forest tree species which has been studied using the CRISPR/Cas9 genome editing technique. Fan et al. (2015) demonstrated efficient CRISPR/Cas9-mediated targeted mutagenesis in *PtoPDS* in the first generation of *Populus tomentosa*. Zhou et al. (2015) also reported a successful application of CRISPR/Cas9 to *Populus tremula* × *P. alba*. They

targeted the 4-coumarate:CoA ligase (*4CL*) gene family, resulting in 100% mutational efficiency for two *4CL* genes targeted. In addition to proof-of-concept, CRISPR/Cas9 has been utilized in *Populus* for elucidating gene functions by creating loss-of-function mutants in *PtoMYB156* and *MYB115*, involved in secondary cell wall biosynthesis and proanthocyanidin biosynthesis, respectively (Wang et al. 2017a; Yang et al. 2017).

Given its successful application in the plants, I believe CRISPR/Cas9 can also be exploited to manipulate the black ash genome. My goal is to generate reproductive sterility in black ash by destroying flower control genes such as *AGAMOUS*, as suggested by previous research.

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CHAPTER 2. ADVENTITIOUS SHOOT REGENERATION FROM IN VITRO LEAF EXPLANTS OF *FRAXINUS NIGRA*

2.1 Abstract

Black ash (*Fraxinus nigra*) is an endangered hardwood tree species under threat of extirpation by the emerald ash borer (EAB), an aggressive exotic phloem-feeding beetle. We have developed an efficient regeneration system through adventitious shoot organogenesis in *F. nigra* using in vitro-derived leaf explants. Two types of leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of plant growth regulators to induce callus and adventitious shoot bud formation. Significant effects of explant, and plant growth regulator interactions were found. The frequency of callus formation ranged from 77.8-94.4% and 88.9-100% from single leaflets and intact compound leaves, respectively, with no significant difference between treatments. For adventitious shoot bud induction, however, 22.2 µM 6-benzylaminopurine (BA) combined with 31.8 µM thidiazuron (TDZ) was the best treatment regardless of the initial leaf explant type, showing 21.1% and 28.8% shoot bud induction, with 1.5 and 1.9 adventitious shoots per explant, from single leaflets and intact compound leaves, respectively. The regenerated shoot buds were elongated on MS medium supplemented with Gamborg B5 vitamins plus 2 mg L^{-1} glycine (MSB5G), 13.3 µM BA, 1 µM indole-3-butyric acid (IBA), and 0.29 µM gibberellic acid. The elongated shoots were continuously micropropagated through nodal stem sectioning until used for rooting. An average of 85.2% of the microshoots were successfully rooted in woody plant medium containing 5.7 µM indole-3-acetic acid plus 4.9 µM IBA with a 10-day initial dark culture, followed by culture under a 16-h photoperiod. Rooted plantlets were acclimatized to the greenhouse and showed normal plant growth and development with 100% survival. This regeneration protocol would be useful for mass propagation for conservation of *F. nigra* and for use in genetic transformation for EAB resistance.

Keywords: Adventitious shoots, black ash, *Fraxinus*, leaf explants, organogenesis, tissue culture

2.2 Introduction

Black ash (*Fraxinus nigra* Marsh.) is a hardwood tree species in North America with a native range in wetland forests from Newfoundland west to Manitoba, south to Indiana and West Virginia (Wright and Rauscher 1990). The strongly ring-porous wood is preferred by Native Americans for making splints for basketry, and also used commercially for furniture, veneer, pulpwood, and non-timber forest products (Benedict and Frelich 2008). Black ash is ecologically valuable as the seeds are consumed by a number of birds and mammals, while twigs and foliage are eaten by white-tailed deer and moose (Anderson and Nesom 2003). While most of the urban and residential ash trees are predominantly white and green ash (*F. americana* L. and *F. pennsylvanica* Marsh.) (Kovacs et al. 2010), black ash inhabits wetland forests and is integral to riparian ecosystems (Nisbet et al. 2015). However, the emerald ash borer (EAB; *Agrilus planipennis* Fairmaire), an aggressive exotic phloem-feeding beetle from Asia, has destroyed tens of millions of ash trees in the United States since the first detection in 2002 in Michigan. EAB is fatal to all native North American ash trees, showing 99% mortality of black, green, and white ash trees with stems greater than 2.5 cm in diameter in forests of southeastern Michigan (Klooster et al. 2014). To date, there is no means to completely eradicate the beetle, and it appears that EAB could functionally extirpate ash in North America with a huge economic and ecological loss (Poland and McCullough 2006; Herms and McCullough 2014). According to a modeling study conducted by Iverson et al. (2016), climate change along with the devastating short-term effects of EAB offered a bleak prospect for the continued existence of black ash in Minnesota.

In vitro plant regeneration is a powerful tool for germplasm conservation of endangered plant species (Jin et al. 2014; Slazak et al. 2015; Wang et al. 2014). Several features of black ash, such as irregular seed production intervals, embryo immaturity at seed set, and complex stratification and germination requirements, make the use of in vitro regeneration technology more feasible (Benedict and David 2003; Gucker 2005; Vanstone and LaCroix 1975). This technology is also useful for production of important secondary metabolites, and a pre-requisite for genetic transformation to confer a new trait such as EAB-resistance. Adventitious shoot regeneration has been established in a number of ash species including white ash (Bates et al. 1992; Palla and Pijut 2011), green ash (Du and Pijut 2008), common ash (*F. excelsior*) (Mockeliunaite and Kuusiene 2004), narrowleaf ash (*F. angustifolia*) (Tonon et al. 2001), pumpkin ash (*F. profunda*) (Stevens and Pijut 2012), and black ash (Beasley and Pijut 2013), using various seed-derived organs such

as hypocotyls and cotyledons. But there are no reports on adventitious shoot regeneration from leaf explants and regeneration of whole plants in *Fraxinus*. The ash seed bank was rapidly depleted and no viable ash seeds were found in several Michigan sites following invasion by EAB (Klooster el al. 2014), indicating limited availability of the use of seed-derived materials. Thus, there is a great need to develop an efficient protocol for shoot regeneration from leaf explants. Black ash leaves are deciduous, opposite, pinnately compound with seven to 11 sessile leaflets (Anderson and Nesom 2003). Leaves are more readily available and usually do not produce inhibitory compounds when cultured in vitro, making this type of explant ideal for use in regeneration systems. Furthermore, development of an in vitro regeneration protocol using leaf explants would be useful to establish a genetic transformation system for multiple gene manipulation via gene stacking. The present study was designed to establish an efficient protocol for adventitious shoot regeneration from in vitro leaf explants of black ash.

2.3 Materials and Methods

2.3.1 Plant material and culture medium

In vitro shoot cultures of black ash (established from open-pollinated seed, National Tree Seed Centre, Fredericton, New Brunswick, Canada) were maintained in Magenta^{TM} GA-7 vessels (Magenta Corp., Chicago, IL) containing a modified Murashige and Skoog (1962) (MS) basal medium (M499; PhytoTechnology Laboratories, Shawnee Mission, KS) with Gamborg B5 vitamins (Gamborg et al. 1968) plus 2 mg L^{-1} glycine (MSB5G), supplemented with 13.3 µM 6-benzylaminopurine (BA), 1 µM indole-3-butyric acid (IBA), 0.2 g L^{-1} casein hydrolysate, and 0.29 µM gibberellic acid (GA_3) (Beasley and Pijut 2013). Unless noted otherwise, all media contained 3% (w/v) sucrose and 0.7% (w/v) Bacto agar (No. 214030; Becton Dickinson and Co., Sparks, MD) with the pH adjusted to 5.7 before autoclaving for 20 min at 121°C. Cultures were maintained in a growth room at 24 ± 2°C under a 16-h photoperiod at approximately 80 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. The in vitro shoots were regularly subcultured to fresh medium every 4 weeks, and micropropagated by nodal stem sectioning.

2.3.2 Effect of explant type and plant growth regulator on callus formation and shoot bud induction

The whole compound leaf (five leaflets attached; Fig. 2.1A) and single leaflets (Fig. 2.1B) were used as explants. Leaf explants obtained from 4-week old in vitro cultures (after micropropagation) were transversely cut two or three times across the midrib and cultured with the abaxial surface in contact with the shoot bud induction medium [MSB5G medium supplemented with 0.5 µM IBA, 10% (v/v) coconut water (C195; *PhytoTechnology* Laboratories), plus BA and thidiazuron (TDZ)]. To study the effect of different concentrations of plant growth regulators (PGRs) on callus formation and adventitious shoot bud induction, we tested 0, 22.2, 26.2, 31.1, or 35.5 µM BA in combination with 27.2 or 31.8 µM TDZ (selected by preliminary factorial experiments with different concentrations of BA (0-35.5 µM) and TDZ (0-36.3 µM); data not shown). Three replicates of 12-15 leaflets or compound leaves each were cultured for each treatment. Cultured leaf explants were incubated in the dark at 26 ± 2°C for 3 weeks, and then transferred to 80 µmol m^{-2} s^{-1} light intensity for culture one additional week before evaluating the frequency of callus formation and adventitious shoot bud induction. Explants forming callus were then transferred to MS medium containing 13.3 µM BA, 4.5 µM TDZ, 0.05 g L^{-1} adenine hemisulfate, and 10% coconut water. After an additional 3 weeks the number of shoots per explant was recorded.

2.3.3 Adventitious shoot elongation, rooting, and acclimatization

Once adventitious shoot buds were initiated, shoot elongation, rooting, and acclimatization followed our previous protocol (Beasley and Pijut 2013). Briefly, all explants initiating shoot buds were transferred to MSB5G medium supplemented with 6.7 µM BA, 1 µM IBA, and 0.29 µM GA_3 in Magenta™ GA-7 vessels for 3 weeks. Cultures were then transferred to MSB5G medium with 13.3 µM BA, 1 µM IBA, 0.2 g L^{-1} casein hydrolysate, and 0.29 µM GA_3. Elongated shoots were excised from leaf explants, subcultured every 4 weeks to fresh medium, and micropropagated through nodal stem sectioning. Elongated microshoots (3-4 cm) were induced to form roots on woody plant medium (WPM; Lloyd and McCown 1980) supplemented with 5.7 µM indole-3-acetic acid (IAA) and 4.9 µM IBA in Magenta™ GA-7 vessels (Beasley and Pijut 2013). Three replications with nine microshoots each were conducted to verify our previous protocol. Microshoots on root induction medium were incubated in the dark at 26 ± 2°C for 10 days and

then transferred to a 16-h photoperiod (80 µmol m^{-2} s^{-1}). After 6 weeks on root induction medium, the frequency of root formation, number of roots and lateral roots per microshoot, and length of roots were evaluated. Rooted plantlets were acclimatized to the greenhouse as described by Beasley and Pijut (2013).

2.3.4 Statistical analysis

Data were analyzed using SPSS 23.0 statistical software (IBM-SPSS 2015). The mean with standard error (± SE) was presented. The percent callus formation, shoot bud induction, and number of shoots were subjected to analysis of variance (ANOVA). Significant difference between treatments was tested by a Duncan's multiple comparison test (p=0.05). The effects of explant type, BA, and TDZ and their interactions were examined using a three-way ANOVA.

2.4 Results and Discussion

2.4.1 Effect of explant type and plant growth regulator on callus formation and shoot bud induction

In the present study, we developed the first protocol on plant regeneration from leaf explants of *F. nigra*. Although several studies have reported regeneration protocols for *Fraxinus* spp. using seed-derived explants such as hypocotyls or cotyledons, there has been no study using leaf explants. In vitro leaf explants were used in this study because leaves often show a better regeneration potential than explants derived from mature tissue (Harding et al. 1996). Furthermore, in vitro leaf explants are more feasible for use in genetic transformation studies because they are aseptic, and gene stacking techniques would be feasible.

We initially tested leaf explants on the best adventitious shoot induction medium (MS medium supplemented with 13.3 µM BA and 4.5 µM TDZ) previously developed in our laboratory with black ash hypocotyl explants, but no response for callus formation and shoot bud induction was obtained (data not shown). We then optimized adventitious shoot regeneration for leaf explants using a combination of BA and TDZ at various concentrations. After the first 4 weeks on shoot bud induction medium, the first visible change was the enlargement in size of leaf explants with callus formation on the cuts in midrib and the petiole base (Fig. 2.1C and D). Through these cut edges more nutrients and PGRs could be absorbed efficiently from the induction medium, as proposed by Sarwar and Skirvin (1997). Most explants produced callus, with the frequency of

callus formation ranging from 77.8-94.4% and 88.9-100% from single leaflet and compound leaf, respectively (Table 2.1). Average percent callus formation was 87.5 ± 1.9 and 94.5 ± 1.4 from single leaflet and compound leaf, respectively, with a significant effect of explant type on callus formation (F=8.74, p<0.01; Table 2.1, 2.2). A three-way ANOVA revealed a significant interaction between BA and TDZ on percent callus formation (Table 2.2).

Visible protuberances and multiple outgrowths which subsequently developed into adventitious shoot buds were observed (Fig. 2.2A-C). Most of the shoot buds developed from callus formed on the abaxial side of the leaf in contact with the medium (Fig. 2.2D), but some shoot buds developed on the adaxial side (Fig. 2.2E) or from callus formed on the petiole base (Fig. 2.2F). This result was similar with those of Pérez-Tornero et al. (2000), who reported most adventitious buds originated from the leaf tissue of apricot (*Prunus armeniaca* L.) in contact with the medium. Different regeneration responses also have been observed in European beech (*Fagus sylvatica* L.) leaf explants, with better shoot formation from proximal half-leaves than distal leaf explants that might be a result of differentials of endogenous hormone transport and maturity between the distal and proximal leaf tissues (Vieitez and San-José 1996). Whereas, petioles were reported to be an excellent explant for adventitious shoot regeneration in several woody plants (Bergmann and Moon 1997; Mohammed et al. 2015). The regeneration response of princess tree (*Paulownia tomentosa* (Thunb.) Siebold and Zucc. ex Steud.) and dragon tree (*P. fortune* (Seem.) Hemsl.) were stimulated by the presence of the leaf lamina along with the attached petiole as explants, compared to using intact petioles only, suggesting the promotive effect of leaf lamina through the establishment of a gradient of diffusible factors (Corredoira et al. 2008; Kumar et al. 1998). Similarly, a possible explanation for our observation (significantly higher frequency of callus formation and shoot bud induction from compound leaf compared to single leaflet; Table 2.2) might be because of a promotive effect of attached leaflets through enhanced transportation of endogenous phytohormones or uptake of PGRs from the medium. We also found that compound leaves were more feasible initial starting materials than single leaflets in terms of being simple and easy to handle.

Although adventitious shoot buds were observed from all BA and TDZ concentrations tested in our study, the response of leaf explants was variable based on the relative concentrations of the two PGRs. The percent of explants with shoot bud induction ranged from 7.9-21.1% in single leaflet, while it varied from 11.7-28.8% in the compound leaf (Table 2.1). Average percent

shoot bud induction was 15.9 ± 1.7 and 20.8 ± 1.3 from single leaflets and compound leaves, respectively, showing a significant effect of explant type on shoot bud induction (F=5.49, p<0.05; Table 2.1, 2.2). The combination of 22.2 µM BA and 31.8 µM TDZ gave the best results on shoot bud induction from both single leaflet and compound leaf. There was no significant difference between treatments for mean number of adventitious shoots per explant using single leaflet; ranged from 1 ± 0.6 to 1.8 ± 1.4 (Table 2.1). However, the combination of 22.2 µM BA and 31.8 µM TDZ proved to produce a significantly higher number of adventitious shoots (1.9 ± 0.5 shoots per explant) when using the compound leaf (Table 2.1). There was no significant effect of explant on mean number of adventitious shoots (Table 2.2).

The concentration of BA played a key role in determining shoot bud induction, showing a significantly lower frequency of shoot bud induction on medium containing only 27.2 µM TDZ (Table 2.1). This result was consistent with the observation that shoot bud induction capacity of physic nut (*Jatropha curcas*) leaf-discs was reduced in the absence of BA (Deore and Johnson 2008). The same BA concentration (22.2 µM) was also found to be successful for shoot formation from hypocotyl explants of black ash with the highest frequency (62.5%) of shoot formation on medium with BA plus 2.3 µM TDZ (Beasley and Pijut 2013). Efficiency of BA over other cytokinins was found in tamarillo (*Cyphomandra betacea*) shoot regeneration from leaf explants, showing more microshoot regeneration with BA treatment compared to TDZ treatment (Kahia et al. 2015). Similarly, high BA concentration efficiently induced multiple bud formation from explants of Cavendish banana (*Musa* spp.) (Subramaniam et al. 2008). However, some contrary results were reported that TDZ was more effective than BA in inducing shoot buds on leaf explants of European beech (Vieitez and San- José 1996), apricot (Pérez-Tornero et al. 2000), and blackberry (*Rubus* hybrid) (Gupta and Mahalaxmi 2009). Rathore et al. (2016) suggested that differential responses of explants caused by different cytokinins may be a result of their varied translocation rates, differential uptake, various effects on metabolic processes, and ability to change the level of endogenous cytokinins.

In this study, a significant interaction was found between BA and TDZ on shoot bud induction (F=3.51, p<0.05; Table 2.2). The higher concentration of TDZ (31.8 µM) in combination with 22.2 µM BA produced more shoot buds, while there was negative correlation between TDZ concentration and shoot bud induction in combination with BA concentration higher than 22.2 µM (Table 2.1). Negative effects of over-abundance of TDZ were reported in pumpkin ash adventitious

shoot formation, showing a decreased percent shoot formation with TDZ concentrations higher than 4.5 µM in combination with BA (Stevens and Pijut 2012). Lower concentrations of TDZ were reported to produce a better response in callus formation from leaf explants of Indian sandalwood (*Santalum album* L.) as higher concentrations were toxic to the explants and caused browning (Singh et al. 2013).

TDZ is well known as a multidimensional PGR which may have both auxin- and cytokinin-like effects, inducing diverse morphogenic responses (Guo et al. 2011). Although cytokinin-like activity of TDZ is well documented, a role of TDZ as a modulator of auxin metabolism has been suggested in several reports of TDZ-induced somatic embryogenesis which is a response commonly associated with auxins (Murthy et al. 1998). Increases in the level of IAA and its precursor, tryptophan, were observed in response to TDZ treatment that caused stimulation of de novo synthesis of auxins in peanut (*Arachis hypogaea* L.) (Murthy et al. 1995). In this study, we obtained good callus formation and shoot regeneration without exogenous auxin application, suggesting black ash leaf explants may contain sufficient levels of endogenous auxin or TDZ may be involved in auxin metabolism to stimulate auxin synthesis. In addition, the dark treatment may influence the levels of endogenous auxin contributing to the induction process (Miguel et al. 1996). Shoot bud browning followed by deterioration was observed when the explants were continuously cultured on the induction medium for more than the first 4 weeks (data not shown). This may be a result of adverse effects of continuous high concentration of cytokinins.

2.4.2 Adventitious shoot elongation, rooting, and acclimatization

The regenerated shoot buds were cultured on MS medium with a lower concentration of BA (6.7 µM) plus 1 µM IBA and 0.29 µM GA_3, but without TDZ to continue adventitious shoot bud enhancement (Fig. 2.3A). While TDZ is a powerful inducer of shoot organogenesis in woody plants, various effects of TDZ on explants and shoots in tissue culture have been reported, including excessive callus formation, bushy shoots, and inhibiting shoot elongation (Beasley and Pijut 2013; Chalupa 1988; Huetteman and Preece 1993). A two-stage culture procedure consisting of a TDZ-treatment of explants followed by TDZ-free cultivation proved efficient in regeneration of *Rhododendron sichotense* with the highest frequency of shoot regeneration along with maximum number of shoots per explant (Zaytseva et al. 2016). In addition to removing TDZ, lowered BA was necessary in the medium for black ash regeneration from hypocotyl explants to

continuously enhance shoot buds (Beasley and Pijut 2013). Rathore et al. (2016) also found that continuous high level of BA produced hyper-hydration in subculture of regenerated Paneer dodi (*Withania coagulans* Dunal) shoot buds, causing adverse effects on the growth and regeneration potential of cultures. A significant elongation of microshoots was obtained on MS medium with lowered BA (from 4.44 to 1.11 µM) (Rathore et al. 2016). However, for routine elongation of shoots regenerated from black ash hypocotyl explants, the concentration of BA needed to be increased after a lower exposure (from 6.7 µM to 13.3 µM) (Beasley and Pijut 2013). After 3 weeks on the shoot bud enhancement medium (Fig. 2.3B), regenerated shoots were cultured on shoot elongation medium with increased BA (13.3 µM) along with 0.2 g L^{-1} casein hydrolysate. When shoots had reached 3-4 cm in height with several nodes (Fig. 2.3C), micropropagation was routinely achieved through nodal stem sectioning until we obtained an adequate number of microshoots for rooting.

Elongated shoots with two or three nodes were rooted on WPM supplemented with 5.7 µM IAA and 4.9 µM IBA (Fig. 2.3D). Callus formation was first observed at the basal end of the shoot, and roots developed from the callus 2 weeks after culture on root induction medium. We achieved 85.2% rooting with a mean of 5.6 ± 0.4 roots per shoot, with a mean root length of 2.6 ± 0.2 cm, and a mean of 2 ± 0.6 lateral roots per shoot (Table 2.3). Twenty-five rooted plantlets with well-developed roots were transferred to pots and acclimatized in the culture room. Normal growth was observed 2-3 weeks after acclimatization (Fig. 2.3E), and plants were then moved to the greenhouse. After an additional 4 weeks, plants were transplanted to larger pots for further growth. One-hundred-percent of the regenerated black ash plants survived in the greenhouse with no morphological abnormalities (Fig. 2.3F). Our laboratory also reported 93% rooting with 4.1 roots per shoot using this rooting procedure for black ash shoots regenerated from hypocotyls (Beasley and Pijut 2013).

2.5 Conclusions

We developed a useful protocol for complete plant regeneration of *F. nigra* via adventitious shoot formation from callus using leaf explants. This protocol will provide the basis for the further applications such as black ash conservation, mass propagation, as well as experimental studies to produce transgenic F. nigra, especially to introduce multiple traits of interest by gene stacking.

Table 2.1 Effect of 6-benzylaminopurine (BA) and thidiazuron (TDZ) on callus formation and adventitious shoot regeneration from two types of in vitro leaf explants of *Fraxinus nigra*.

PGR (µM)		Callus formation (%)		Shoot bud induction (%)		Mean No. shoots	
BA	TDZ	Single leaflet	Compound	Single leaflet	Compound	Single leaflet	Compound
0	27.2	77.8 ± 2.8a	88.9 ± 5.1a	7.9 ± 3.7b	11.7 ± 3.9b	1.3 ± 0.9a	0.5 ± 0.2b
22.2	27.2	83.3 ± 12.7a	89.3 ± 9.1a	13.4 ± 3.9ab	14.3 ± 2.6ab	1.0 ± 0.6a	0.9 ± 0.2ab
26.2	27.2	91.7 ± 4.8a	94.7± 5.3a	21.1 ± 2.0a	25.7 ± 5.9ab	1.8 ± 0.4a	1.3 ± 0.2ab
31.1	27.2	94.4 ± 5.6a	97.3 ± 2.7a	20.6 ± 4.2a	24.1 ± 3.6ab	1.8 ± 0.2a	1.7 ± 0.7ab
35.5	27.2	94.4 ± 2.8a	100.0 ± 0a	20.5 ± 2.3a	28.3 ± 3.4a	1.4 ± 0.2a	1.2 ± 0.2ab
0	31.8	86.1 ± 5.6a	98.7 ± 1.3a	12.8 ± 2.8ab	18.5 ± 5.0ab	1.8 ± 1.4a	1.1 ± 0.4ab
22.2	31.8	91.7 ± 4.8a	100.0 ± 0a	21.1 ± 3.5a	28.8 ± 3.3a	1.5 ± 0.3a	1.9 ± 0.5a
26.2	31.8	80.6 ± 10.0a	92.0 ± 3.9a	17.1 ± 2.8a	21.9 ± 7.2ab	1.7 ± 0.7a	1.5 ± 0.2ab
31.1	31.8	86.1 ± 2.8a	90.7 ± 4.5a	12.7 ± 2.7ab	17.2 ± 5.9ab	1.8 ± 1.4a	1.0 ± 0.4ab
35.5	31.8	88.9 ± 2.8a	93.3 ± 3.7a	12.4 ± 2.9ab	17.9 ± 5.2ab	1.7 ± 1.2a	0.9 ± 0.3ab
Average		87.5 ± 1.9	94.5 ± 1.4	15.9 ± 1.7	20.8 ± 1.3	1.6 ± 0.2	1.2 ± 0.2

Mean ± SE followed by the same letter in same column were not significantly different by the Duncan's multiple comparison test ($p < 0.05$)

Table 2.2. Summary of three-way ANOVA results for examining the effect of each treatment and their interactions.

	df	Callus formation		Shoot bud induction		No. of shoots	
		F	*p*	*F*	*p*	*F*	*p*
Explant (E)	1	8.74**	0.004	5.49*	0.022	2.21	0.143
BA (B)	4	0.81	0.522	2.06	0.097	0.38	0.822
TDZ (T)	1	0.03	0.869	0.09	0.766	0.68	0.414
E × B	4	0.34	0.848	0.05	0.996	0.35	0.844
E × T	1	0.29	0.592	0.11	0.743	0.01	0.915
B × T	4	2.79*	0.034	3.51*	0.012	0.63	0.644
E × B × T	4	0.11	0.978	0.13	0.972	0.24	0.914

Statistical significance at *$p < 0.05$; **$p < 0.01$

6-benzylaminopurine (BA) and thidiazuron (TDZ)

Table 2.3. In vitro root formation from microshoots of *Fraxinus nigra* regenerated from leaf explants.

Replicate	Rooting (%)	Mean No. roots per shoot	Mean No. lateral roots per shoot	Mean root length (cm)
1	77.8	6.3 ± 0.7a	2.7 ± 1.1a	2.6 ± 0.4a
2	100	4.9 ± 0.6a	1.6 ± 0.8a	2.5 ± 0.3a
3	77.8	5.3 ± 0.7a	1.7 ± 1.6a	2.6 ± 0.4a
Mean	85.2	5.6 ± 0.4	2.0 ± 0.6	2.6 ± 0.2

Mean ± SE for nine microshoots per replicate

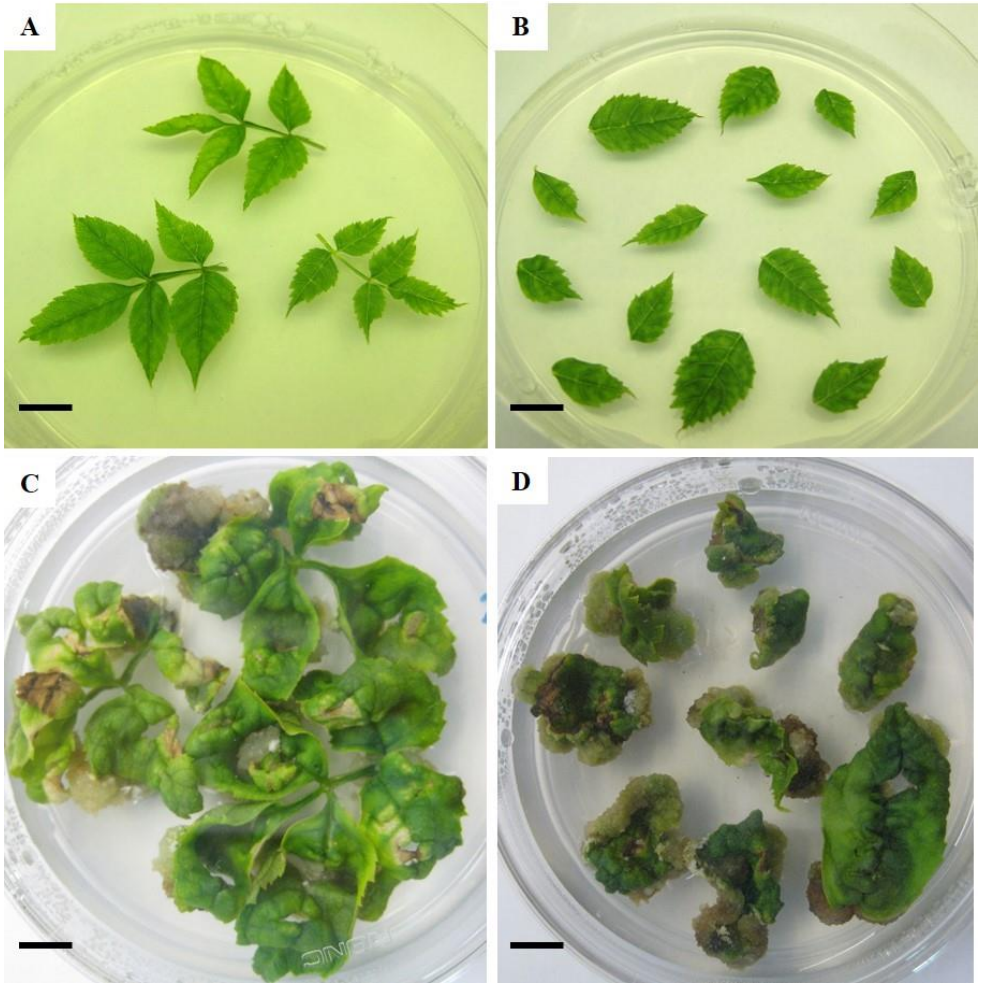

Figure 2.1 Callus formation from leaf explants of *Fraxinus nigra* (black ash). Compound leaves with five leaflets attached (A) or single leaflets (B) were placed on induction medium. Each leaf was transversally cut two or three times across the midrib and cultured with the abaxial surface in contact with the medium. (C, D) Callus was induced from the cuts on the abaxial side and petiole ends after 4 weeks (3 weeks in the dark followed by 1 week in the light) (*bar* = 1 cm).

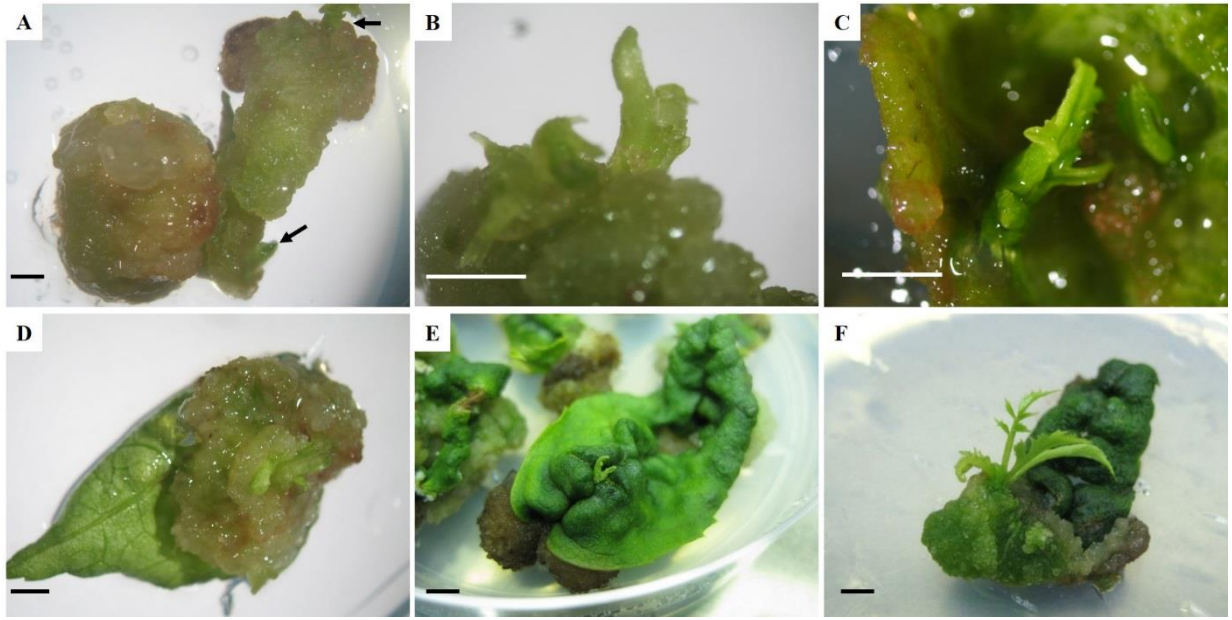

Figure 2.2 Adventitious shoot bud initiation. (A) Protuberance (arrow) development and (B, C) Adventitious shoot bud initiation (*bar* = 1 mm). Adventitious shoot buds arising from callus formed on the abaxial side (D), adaxial side (E), and on the petiole (F) after 4 weeks on MSB5G medium with 22.2 µM BA, 31.8 µM TDZ, and 0.5 µM IBA (*bar* = 2 mm).

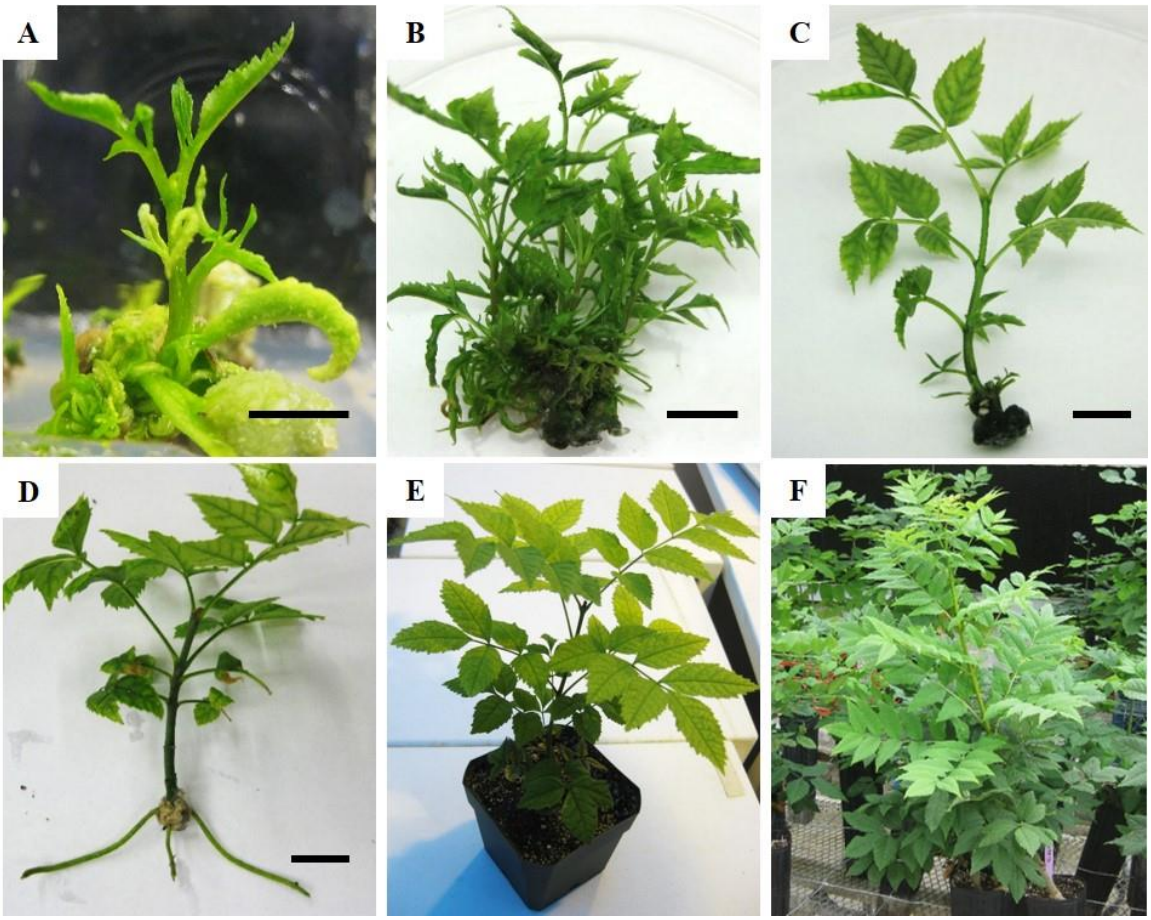

Figure 2.3 Leaf-explant derived shoot regeneration of *Fraxinus nigra* (black ash). (A) Adventitious shoot on shoot induction medium (*bar* = 0.5 mm); (B) Shoots on shoot bud enhancement medium (*bar* = 1 cm); (C) Microshoot elongating on shoot elongation medium (*bar* = 1 cm); (D) In vitro root production (*bar* = 1 cm); (E) Acclimatization of a rooted plantlet in the culture room; and (F) Acclimatized black ash plant in the greenhouse.

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CHAPTER 3. OPTIMIZATION OF *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION OF *FRAXINUS NIGRA* AND DEVELOPMENT OF BLACK ASH FOR EMERALD ASH BORER-RESISTANCE

3.1 Abstract

Emerald ash borer (EAB; *Agrilus planipennis* Fairmaire) is the most devastating insect pest of North American ash species, including black ash (*Fraxinus nigra* Marsh.). As a first step in an effort to develop transgenic black ash plants resistant to EAB, we successfully established an efficient *Agrobacterium*-mediated transformation system for black ash hypocotyls. Kanamycin and timentin at 40 mg L^{-1} and 300 mg L^{-1}, respectively, were most effective to select transformed explants and control excess *Agrobacterium* growth. Using a plant transformation vector harboring the enhanced green fluorescent protein (*eGFP*) gene, the effects of *Agrobacterium* strain, bacterial density, and the concentration of Silwet L-77 on transformation efficiency were evaluated. The best result was obtained when *Agrobacterium* strain EHA105 was used at a density of OD_{600} = 1.0. Silwet L-77 failed to promote transformation frequency and showed an adverse effect at higher concentrations (>0.015%). Using this optimized transformation system, transgenic black ash shoots expressing a synthetic *Bacillus thuringiensis* toxin gene (*cry8D2*) were regenerated. Although no morphological abnormality was observed, transgenic shoots showed severe growth retardation. Three independent transgenic lines were selected for further assessment. All selected lines contained two copies of the *cry8D2* gene, and the expression of the transgene was verified in transcript and protein levels. These transgenic shoots can be used for future bioassay to evaluate its efficacy against EAB.

Keywords: *Agrobacterium*-mediated transformation, *Bacillus thuringiensis*, biotechnology, black ash, emerald ash borer, insect resistance.

3.2 Introduction

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is an invasive wood-boring insect native to Asia (China, Japan, Korea, Mongolia, and eastern Russia) that is threatening North American native ash trees; first identified in Michigan in 2002. Although a tremendous effort has been made to contain the nascent infestation, EAB has spread rapidly through natural dispersal and human-assisted firewood movement, and as of September 2017, it was found in 31 states, and the Canadian provinces of Windsor and Quebec (http://www.emeraldashborer.info). Indeed, the EAB is the single most destructive insect invasion in North America, often being compared to the chestnut blight, a devastating fungal disease that destroyed 3.5 billion American chestnut trees in the early 1990s. It appears likely that approximately 9 billion ash trees are potentially at risk of extirpation nationally (Mercader et al. 2009; Muirhead et al. 2006).

Black ash (*Fraxinus nigra* Marsh.) is one of the highly susceptible ash species, and is native to northeastern North America and eastern Canada. In addition to the economic impact of black ash mortality caused by EAB to the timber industry (flooring, millwork, and crates), the loss of black ash could have devastating impacts ecologically resulting in dramatic shifts in riparian ecosystem structure and function, as black ash is the dominant species in wetland forests (Ellison et al. 2005; Telander et al. 2015). Black ash wood is also preferred by Native Americans for making splints for basketry. This is a significant component of cultural and historical identity as well as a good income source, and now this tradition is under threat (Herms and McCullough 2014; Willow 2011).

Currently, a number of management strategies have been implemented to combat EAB including insecticide options (Poland et al. 2016) and biocontrol using parasitoids (USDA–APHIS/ARS/FS 2017). Molecular approaches have also been studied with Manchurian ash (*F. mandshurica*), resistant Asian ash species sharing a co-evolutionary history with EAB, to find endogenous factors that may confer resistance (Bai et al. 2011; Cipollini et al. 2011; Whitehill et al. 2012). Genetic engineering, meanwhile, could be an excellent alternative to control EAB and conserve ash trees through the introduction and expression of an insecticidal δ-endotoxin gene (*cry* gene) from *Bacillus thuringiensis* (*Bt*) into the ash genome. Attempts have been made to develop *Bt*-transgenic plants, and insect-resistance was successfully established in various plant species, not only for agricultural crops but also for a few forest trees (Génissel et al. 2003; Harcourt et al.

2000; Lachance el al. 2007; Shin et al. 1994; Tang and Tian 2003). Among more than 750 different *Bt* genes, the Cry8Da toxin produced by *Bt* subsp. *galleriae* SDS-502 strain was tested and its efficacy was confirmed against the EAB (Bauer and Lodoño 2011).

Agrobacterium-mediated genetic transformation is widely used as a tool for the introduction of foreign genes into plants. Many tree species, however, have proven to be recalcitrant to genetic modification showing relatively low transformation frequency and regeneration rate. Thus, the transformation conditions need to be optimized for a particular plant species. Previous reports have demonstrated that *Agrobacterium*-mediated transformation was successful for other *Fraxinus* spp. including white ash (*F. americana*) (Palla and Pijut 2015), green ash (*F. pennsylvanica*) (Du and Pijut 2009; Roome 1992), and pumpkin ash (*F. profunda*) (Stevens and Pijut 2014), but no transformation system has been developed for black ash. Therefore, the main goal of this research was to develop an efficient *Agrobacterium*-mediated transformation protocol for black ash. Our previously developed methods for in vitro regeneration of black ash were used as a basis for regeneration of putative transformants (Beasley and Pijut 2013; Lee and Pijut 2017a). Various factors affecting transformation efficiency were evaluated to optimize the procedure. We then used a synthetic, full-length *cry8D2* gene and its expression in transgenic shoots was confirmed by real-time polymerase chain reaction (qPCR) and Western blot analysis. We herein describe the first production of genetically modified *Bt*-ash lines that can be used for future bioassays to evaluate their efficacy against the EAB.

3.3 Materials and Methods

3.3.1 Plant material

Mature black ash seeds were obtained from the National Tree Seed Centre (Natural Resources Canada, Fredericton, New Brunswick, Canada) and stored in sealed bags at 4°C in the dark until used. Seeds were disinfested and stratified as described by Beasley and Pijut (2013), mature embryos were extracted and cultured horizontally on a modified Murashige and Skoog (1962) (MS; M499, *Phyto*Technology Laboratories, Shawnee Mission, KS) pre-culture medium with organics (100 mg L^{-1} myoinositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCL, 0.1 mg L^{-1} thiamine HCL, and 2 mg L^{-1} glycine), and supplemented with 13.3 µM 6-benzylaminopurine (BA), 4.5 µM thidiazuron (TDZ), 50 mg L^{-1} adenine hemisulfate, 10% (v/v)

coconut water (C195, *Phyto*Technology Laboratories) in Petri plates (100 × 25 mm; 45 mL medium) to allow germination. Hypocotyls were excised from 7-day-old in vitro seedlings and used to determine explant sensitivity to the antibiotics and for transformation experiments. Unless noted otherwise, all media contained 3% (w/v) sucrose and 0.7% (w/v) Bacto agar (No. 214030; Becton Dickinson and Co., Sparks, MD) with the pH adjusted to 5.7 before autoclaving for 20 min at 121 °C. All cultures were maintained in a growth room at 24 ± 2°C under a 16 h photoperiod (approximately 80 μmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps.

3.3.2 Effect of kanamycin and timentin on hypocotyl explants

The effect of antibiotics on callus induction and shoot formation was investigated to determine the optimum concentration for selection of transformed explants. Seven-day-old hypocotyls were cultured horizontally on MS pre-culture medium with kanamycin (0, 10, 20, 30, 40, or 50 mg L^{-1}) or timentin (0, 100, 200, 300, 400, or 500 mg L^{-1}) in Petri plates (100 × 25 mm; 45 mL medium). Antibiotics were dissolved in sterile, deionized water and filter-sterilized (0.22 μm) and added to the medium after autoclaving. Hypocotyls were cultured for 3 weeks, transferred to fresh treatment medium, and the regeneration response for callus and shoot induction were recorded after 6 weeks of culture in vitro. Three replicates of 12 hypocotyls each were used for each treatment.

3.3.3 Transformation vectors and *Agrobacterium* culture

Based upon previous results in our lab, the pq35GR vector (Fig. 3.1a; Li et al. 2004) that contained a reporter gene encoding enhanced green fluorescent protein (eGFP) driven by the CaMV 35S promoter was utilized to test the various factors affecting transformation efficiency. The pq35GR vector was transformed into two *Agrobacterium tumefaciens* strains, EHA105 and GV3101, to test the effect of *Agrobacterium* strain on transformation efficiency. EHA105 strain harboring pq35GR vector was used for further experiments to test the effects of bacterial density (OD_{600} = 0.5, 1.0, or 1.5) and Silwet L-77 concentration (0, 0.0075, 0.015, or 0.03%).

Single *Agrobacterium* colonies harboring pq35GR vector were inoculated in 20 mL liquid YEP medium (10 g L^{-1} yeast extract, 10 g L^{-1} bactopeptone, and 5 g L^{-1} NaCl, at pH 7.0) in the presence of appropriate antibiotics (20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin), and cultured in the dark for 2 days at 28°C. Once OD_{600} = 0.9–1.0 was reached, the cells were collected by

centrifugation at 4,500 rpm for 15 min, and the pellet was re-suspended in 20 mL liquid MS co-culture medium (13.3 μM BA, 4.5 μM TDZ, 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water) with the addition of 100 μM acetosyringone. To test the effect of bacterial density on transformation efficiency, *Agrobacterium* grown at stationary phase was harvested and re-suspended in 20 mL liquid MS co-culture medium to adjust a final OD_{600} = 0.5, 1.0, or 1.5. The suspension of *Agrobacterium* was incubated at 28°C for another 2–3 h before being used for co-cultivation with hypocotyl explants. To test the effect of Silwet L-77 concentration on transformation efficiency, Silwet L-77 (0, 0.0075, 0.015, or 0.03%) was added to liquid MS co-culture medium before the vacuum-infiltration.

The vector pBI221-8D2, containing a synthetic, full length Cry8Da protoxin was obtained from Phyllom LLC (Mountain View, CA). In this gene, the ratio between GC and AT contents was modified to increase the GC content from the naturally occurring *Bt cry8Da* sequence (personal communication from Phyllom). This vector was used in a particle-gun transformation system for turf grass (Phyllom). We were not successful in cloning the *cry8Da* gene into our pq35GR vector. We therefore utilized the pBI121 binary vector, excised out GUS, and inserted the *cry8Da2* from pBI221-8D2 downstream of the CaMV 35S promoter. A FLAG tag was added to the N-terminal region of *cry8D2* sequence using *Xba*I and *Bam*HI (Fig. 3.1b). The final expression cassette for Cry8D2 was transformed into *Agrobacterium* strain EHA105.

3.3.4 *Agrobacterium*-mediated transformation and regeneration of putative transformants

Hypocotyls excised from 7-day-old embryos were submerged in 20 mL liquid MS co-culture medium and sonicated for 90 s. Explants were then immersed in *Agrobacterium* suspension and vacuum-infiltrated (62.5 cm Hg) for 10 min. Explants were then blotted dry on sterile Whatman filter paper and placed horizontally on semi-solid MS co-culture medium for 2–3 days in the dark at 28°C. After co-cultivation, the explants were rinsed three times in liquid MS co-culture medium to remove excess *Agrobacterium* prior to being cultured horizontally on MS selection medium (13.3 μM BA, 4.5 μM TDZ, 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water, 40 mg L^{-1} kanamycin, and 300 mg L^{-1} timentin) for 6 weeks. Once adventitious shoot buds were induced from putative transformed explants, the kanamycin-resistant explants were cultured on selection medium without TDZ in order to prevent shoot elongation inhibition. Once the

insertion of *cry8D2* transgene was confirmed by PCR, the transgenic shoots were regularly subcultured to fresh medium without kanamycin every 3 weeks.

3.3.5 Visualization of enhanced green fluorescent protein

The expression of GFP in the explants was monitored by using a microscope (Nikon Eclipse C*i*, Nikon Instruments Inc., Melville, NY) equipped with a GFP3 filter (excitation 470/40, emission 525/50 nm). Leaves from non-transformed control explants and putative transgenic explants were examined to compare the presence or absence of green fluorescence. Images were photographed with a digital camera, and GFP-positive explants were counted from the total transformed explants to evaluate transformation efficiency.

3.3.6 Molecular analysis of transgenic lines

3.3.6.1 DNA extraction and PCR analysis

Genomic DNA was isolated from the leaves of non-transformed control shoots and the regenerated putative transgenic shoots following the procedure described by Lefort and Douglas (1999) with a slight modification. PCR analysis was performed to specifically amplify a 352-bp fragment of *nptII*, and a 430-bp fragment and 3.5-kb full-length of *cry8D2*. The pBI121-*cry8D2* plasmid was used as a positive control template. The PCR reaction mix was prepared in a total volume of 25 μL containing 1 μL genomic DNA (100–200 ng μL^{-1}), 12.5 μL One *Taq* Hot Start 2× Master Mix (New England Biolabs, Ipswich, MA, USA), 1 μL each of 10 μM nptII_F and nptII_R or 10 μM cry_430_F and cry_430_R or 10 μM cry_Full_F and cry_Full_R primers (Table 3-1), and sterile-deionized water was added to make the final volume. All the cycling programs consisted of an initial denaturation at 94°C for 30 s, followed by 35 cycles of 94°C for 15 s, 55°C for 15 s, 68°C for 30 s for *nptII* and *cry8D2* partial fragments, or 3 min for *cry8D2* full sequence, and a final extension at 68°C for 5 min. Amplified products of the PCR were visualized under UV light after gel electrophoresis (1% w/v agarose plus 0.3 μg mL^{-1} ethidium bromide).

3.3.6.2 RNA extraction and transgene expression analysis

Total RNA was isolated from leaves of non-transformed control shoots and the regenerated putative transgenic shoots using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Isolated 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. To analyze the relative expression of *cry8D2*, semi-quantitative reverse transcription (RT)-PCR and qPCR were conducted. The qPCR was performed as described by Lee and Pijut (2017b) with 20 μL reaction solution containing 1 μL cDNA, 1 μL each of 10 μM cry_qRT_F and cry_qRT_R or 10 μM eEFAα_F and eEFAα_R primers (Table 3-1), 10 μL iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), and sterile-deionized water was added to make the final volume. The PCR reaction was as follows: 95°C for 60 s, followed by 40 cycles of 95°C for 10 s, 57°C for 20 s, 72°C for 30 s, and followed by a melting curve analysis from 65 to 95°C performed with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Relative transcript levels for mRNAs were obtained using the comparative cycle threshold (C_t) method and normalized to translation elongation factor alpha (*eEFAα*) from black ash. Each reaction was repeated three times.

3.3.6.3 Determination of transgene copy number

To determine gene copy number of *cry8D2* in PCR-confirmed transgenic lines, qPCR method was used. Black ash *AGAMOUS* (*FnAG*) was selected as a single-copy endogenous reference gene (Lee and Pijut 2017b). Genomic DNA was extracted as described previously, and used as a template. A 143-bp fragment of *FnAG* and a 99-bp fragment of *cry8D2* were amplified using primers FnAG_qRT_F and FnAG_qRT_R or cry_qRT_F and cry_qRT_R, respectively (Table 3-1). Each reaction contained the following reagents in 20 μL: 2 μL genomic DNA (100 ng μL^{-1}), 1 μL of each primer, 10 μL iTaq™ Universal SYBR® Green Supermix (Bio-Rad), and sterile-deionized water to make the final volume. The PCR reaction was as follows: 95°C for 60 s, followed by 40 cycles of 95°C for 10 s, 57°C for 20 s, 72°C for 30 s, and followed by a melting curve analysis from 65 to 95°C performed with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Each reaction was repeated three times, and the relative standard curve of the serial dilution of genomic DNA was constructed for both the *cry8D2* and reference gene. Gene copy number was estimated by comparing C*t* values of the *cry8D2* with *FnAG*, following the calculation reported by Weng et al. (2004).

3.3.6.4 Western blot analysis

Total protein was extracted from leaves of transgenic lines of which the *cry8D2* transcript expression was confirmed. Plant tissues were ground in liquid nitrogen and re-suspended in extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA pH 8.0, protein inhibitor cocktail, 1 mM PMSF, 2 mM DTT, 0.1% Triton X-100). The total protein from each transgenic line was separated on 8% SDS-PAGE gels (Laemmli, 1970), transferred to a polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific), and immunoblotted with a monoclonal anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA). The membrane probed with antibody was detected using the SuperSignal® West Pico Chemiluminescent Substrate™ according to the manufacturer's protocol (Thermo Fisher Scientific).

3.3.7 Statistical analysis

Data were analyzed using SAS® 9.3 software package (SAS® Institute Inc., 2011). The mean with standard error (±SE) was presented. The percent callus formation and shoot induction, and transformation efficiency were subjected to analysis of variance (ANOVA). Significant difference between treatments was tested by a Duncan's multiple comparison test ($p = 0.05$).

3.4 Results and Discussion

3.4.1 Effect of kanamycin and timentin on hypocotyl explants

To determine the optimal concentration of antibiotics for selection of putative transgenic lines, hypocotyls were exposed to various concentrations of kanamycin and timentin. Kanamycin effectively inhibited callus formation and shoot induction as the concentration was increased, and showed significant inhibition at 30 mg L^{-1} or higher concentration (Table 3-2). Other ash studies reported that 20 mg L^{-1} kanamycin significantly inhibited organogenesis of green and pumpkin ash (Du and Pijut 2009; Stevens and Pijut 2014). Palla and Pijut (2015) found a similar result that 30 mg L^{-1} kanamycin was lethal to white ash shoot organogenesis. Timentin had little influence on black ash hypocotyl regeneration, showing a shoot induction frequency ranging from 40 to 53.3% (data not shown). We determined 40 mg L^{-1} kanamycin and 300 mg L^{-1} timentin to be the optimal concentration for screening transgenic shoots in subsequent transformation experiments, in order

to minimize the potential for escape of non-transformed explants and to ensure only transformed tissues would be regenerated.

3.4.2 Optimization of conditions for efficient transformation of black ash hypocotyls

The transformation conditions such as *Agrobacterium* strain, bacterial density, and the concentration of Silwet L-77 were optimized. The expression of GFP was monitored in leaves regenerated from transformed hypocotyls harboring pq35GR vector to aid in calculation of transformation efficiency (Fig. 3.2).

The effect of two commonly used *Agrobacterium* strains, EHA105 and GV3101, were tested. The rate of GFP positive explants was significantly higher when *Agrobacterium* strain EHA105 was used than using GV3101; it was approximately 2.7-fold higher (Fig. 3.3a). The choice of appropriate *Agrobacterium* strain for a particular plant species is one of the most important factors for successful plant transformation, as different strains show various virulence on host plants, determining the rate of T-DNA transfer (Han et al. 2000; Ko et al. 2003; Song et al. 2011). For European plum (*Prunus domestica* L.), *Agrobacterium* strains LBA4404 and EHA105 overall exhibited higher rates of transformation than GV3101 (Song et al. 2011). Han et al. (2000) found that EHA105 was superior to C58 and LBA4404 for some recalcitrant cottonwood hybrids, although C58 was the most efficient for *Populus nigra* transformation (Confalonieri et al. 1994). Soybean (*Glycine max* L.) cotyledon explants transformed with strains EHA105 and GV3101 showed low or complete inhibition of embryogenic response and no GUS expression, while those transformed with strain KYRT1 showed the highest frequency of embryogenesis and GUS expression (Ko et al. 2003). According to our results, we used strain EHA105 for subsequent transformation experiments for black ash.

The influence of bacterial density on transformation efficiency was investigated. The highest frequency of transformation was obtained with a final bacterial density of OD_{600} = 1.0 (Fig. 3.3b), while the lowest transformation efficiency was observed when OD_{600} = 0.5 was used. Although the transformation efficiency was not statistically different between OD_{600} = 1.0 and OD_{600} = 1.5, *Agrobacterium* overgrowth followed by necrosis was observed for explants cultured in *Agrobacterium* with OD_{600} = 1.5. Transformation of peanut (*Arachis hypogaea*) petioles was optimized at an OD_{600} = 0.6, and exposure to higher concentrations (OD_{600} = 0.8 and 1.0) of bacterial suspension caused contamination of the explants (Liu et al. 2016). The highest

transformation efficiency for hybrid aspen (*Populus tremula* × *P. tremuloides*) was obtained with a final bacterial density of OD_{600} = 1.0, while the transformation yield dropped by half for the OD_{600} = 0.5 and 2.0 (Takata and Eriksson 2012). Although higher density of bacteria should be used for recalcitrant species to ensure increasing bacterial attachment, it may cause hypersensitive physiological response of explants, resulting in decrease of regeneration potential and unsuccessful recovery of transformed cells (Sreeramanan et al. 2008; Yong et al. 2006).

The effect of the surfactant Silwet L-77 concentration was also investigated. Silwet L-77 is often used for *Agrobacterium*-mediated transformation, especially in *Arabidopsis* transformation, replacing the vacuum-infiltration step of the floral dip method. Silwet L-77 allows a bacterial solution to penetrate into plant tissues such as flowers and leaves, enhancing the transformation efficiency. However, necrosis of explants was observed when a high concentration of Silwet L-77 was used (Li et al. 2009), as higher concentrations over 0.02% (v/v) may be toxic (Zhang et al. 2006). Thus, a proper concentration of Silwet L-77 should be determined. In our study, the control group of zero Silwet L-77 showed similar statistical transformation efficiency with 0.0075% Silwet L-77 treatment (Fig. 3.3c). But, with 0.015% Silwet L-77, the transformation efficiency showed a significant decrease (Fig. 3.3c), and necrosis was observed for most hypocotyls transformed in medium containing 0.03% Silwet L-77 (data not shown). The concentration of Silwet L-77 was optimized at 0.005 and 0.02% for *Arabidopsis* seedling cotyledons and flowers, respectively (Li et al. 2009; Zhang et al. 2006). For hybrid aspen leaf explants, Silwet L-77 concentration up to 0.015% was positively correlated with transformation efficiency, but the viability of explants was decreased with 0.03% Silwet L-77 (Takata and Eriksson 2012). Our data suggested that the surfactant Silwet L-77 was not necessary for black ash transformation using hypocotyls, as long as sonication and vacuum-infiltration were applied. Sonication-assisted *Agrobacterium* transformation (SAAT) has been widely applied for various plants to enhance transformation efficiency (Alam et al. 2017; Beranová et al. 2008; Chu et al. 2016), including other ash species (Du and Pijut 2009; Palla and Pijut 2015; Stevens and Pijut 2014). Brief periods of SAAT causes a large number of microwounds throughout the plant tissue, allowing *Agrobacterium* easy access into the target plant cells, even in meristematic tissue buried under several layers (Trick and Finer 1997). Combined with sonication, vacuum-infiltration has been used to increase the rate of T-DNA delivery by enhancing *Agrobacterium* penetration into plant tissue (de Oliveira et al. 2009; Liu et al. 2005; Subramanyam et al. 2011).

Therefore, an efficient transformation procedure consisting of 90-s sonication plus 10-min vacuum-infiltration using *Agrobacterium* strain EHA105 at OD_{600} = 1.0 without the addition of Silwet L-77, was developed for black ash hypocotyl explants.

3.4.3 Regeneration of transgenic black ash shoots expressing *cry8D2*

Using our optimized transformation system, we developed transgenic black ash expressing the insecticidal *Bt-cry* gene. In the present study, we used the *cry8D2* gene, a synthetic version of the natural *cry8Da* gene produced by *Bt* SDS-502 that showed a narrow toxicity spectrum to *Anomala cuprea* (Coleopteran) (Asano et al. 2003), including EAB (Bauer and Londoño 2011). It has been documented that the expression levels were low when wild-type native *cry* genes were transformed into plants, since some features typical of native *cry* genes such as AT-rich nucleotide sequences, poor coding capacity, and cryptic polyadenylation signals reduced transcript stability in plants and resulted in rapid degradation of mRNA encoding the *cry* gene (Diehn et al. 1996; Murray et al. 1991). Thus, our *cry8Da* gene was manipulated for plant usage with increasing GC content. The Cry8D2 expression cassette *35S::FLAG-cry8D2* (Fig. 3.1b) was transformed into black ash hypocotyls, and transformants were selected on MS medium containing 40 mg L^{-1} kanamycin (Fig. 3.4a). A total of 117 kanamycin-resistant hypocotyls survived from a total of 3,350 inoculated explants, giving a transformation frequency of 3.5%. Among the 117 putative transgenic lines, only 23 explants successfully regenerated adventitious shoots (Fig. 3.4b). PCR analysis confirmed the presence of the Cry8D2 expression cassette from 21 individual lines (Fig. 3.4c). However, transgenic shoots generally showed severe growth restriction in comparison to non-transformed shoots, and some PCR-positive transgenic shoots deteriorated followed by necrosis during continuous subculture (data not shown). Regarding the slow growth rate, negative effects of kanamycin were reported on shoot proliferation and elongation of transgenic pumpkin and white ash, even when the transgenic shoots showed kanamycin-resistance conferred by expression of the *nptII* gene (Palla and Pijut 2015; Stevens and Pijut 2014). However, a normal rate of shoot elongation was recovered when the transgenic white ash shoots, of which the presence of transgenes had been confirmed by PCR, were cultured on elongation medium without kanamycin after two to three subcultures (Palla and Pijut 2015). In addition to removal of kanamycin from the medium, application of a liquid medium overlay has been shown to increase the overall growth rate of transgenic pumpkin ash shoots (Stevens and Pijut 2014). Neither of these

steps made any difference on transgenic black ash shoot growth, but initiated excess callus formation. Similarly, liquid medium overlay failed to promote transgenic white ash shoot growth (Palla and Pijut 2015), although it enhanced elongation of non-transformed white ash axillary shoots (van Sambeek et al. 2001).

Some plant transformation studies reported that over-expression of a *Bt-cry* gene showed adverse effects such as significantly delayed plant growth and development (Acharjee et al. 2010; Chakrabarti et al. 2006; Khatodia et al. 2014; Rawat et al. 2011; Sachs et al. 1998) and phenotypic abnormality (Singh et al. 2016). Such detrimental effects in *Bt* transgenic plants might be because of the high-level of expression of Cry toxic protein. Chakrabarti et al. (2006) speculated that high-levels of Cry9aA2 protein accumulation (approximately 10% of the total soluble protein) in tobacco leaves caused the delay in plant development. Acharjee et al. (2010) reported significant reduction in growth rate and seed production of transgenic chickpea (*Cicer arietinum* L.) lines expressing high-levels of Cry2Aa protein. Similarly, transgenic rice expressing Cry1Ac and Cry2A protein at high-level (>1% of total soluble protein) showed stunted growth and sterility, while such defects were not observed when another insecticidal gene (snow drop lectin; *gna*) was expressed at higher levels (up to 2% of total soluble protein) (Gahakwa et al. 2000). However, transgenic tobacco overexpressing *Bt-cry2Aa2* showed normal growth even with higher accumulation of *Bt* insecticidal protein in leaves (45.3% of the total soluble protein) (de Cosa et al. 2001), suggesting that other factors should be considered affecting variation in transgenic plants such as insertional-mutagenesis caused by random-integration of T-DNA, or somaclonal variation (Larkin and Scowcroft 1981; Shu et al. 2002; van Lijsebettens et al. 1991).

Three independent lines (T38, T40-4, and T41-6) were selected that showed stable viability (Fig. 3.5a) for further assessment. PCR analysis was conducted to confirm that the intact *cry8D2* gene (full length of 3.5-kb) was integrated into the genome of these three selected transgenic lines (Fig. 3.5b). Rearranged and/or truncated transgene fragments were often found in other plant transformations (Kohli et al. 2003; Makarevitch et al. 2003; Pawlowski and Somers 1998; Weng et al. 2004), so the integration of the intact transgene should be confirmed to ensure the expression of the functional transgene. We then confirmed the expression of *cry8D2* mRNA driven by CaMV 35S promoter. Although CaMV 35S promoter has been widely used for transgene expression in plants, transgene silencing caused by hyper-methylation in the transgene itself or promoter regions has been reported under the control of CaMV 35S promoter (Gambino et al. 2010; Okumura et al.

2016). Consistent transcriptional silencing was observed in transgenic gentian (*Gentiana triflora* × *G. scabra*) that was induced by cytosine methylation exclusively in the CaMV 35S promoter region, regardless of the copy number and the insertion location of T-DNA (Mishiba et al. 2005). In the present study, we also found that some transgenic lines showed gene silencing or very weak expression of *cry8D2* transgene (Fig. 3.5c; lane 2-4). However, all three selected lines (T38, T40-4, and T41-6) presented a strong *cry8D2* transgene expression, as measured by semi-quantitative RT-PCR (Fig. 3.5c). The relative transcript expression levels varied among three lines: approximately 2.5-fold higher expression in T38 and T41-6 compared to T40-4 (Fig. 3.5d). Western blot analysis using total protein extracts from leaves presented the expression of Cry8D2 protein with approximately 130 kDa, although the expression levels were different among the lines (Fig. 3.5e). T38 expressed the highest level of Cry8D2 protein production, while T40-4 produced more protein than T41-6, that was not consistent with the transcript expression levels (Fig. 3.5 d, e). A similar inconsistent pattern between the level of mRNA and the amount of protein was reported in one transgenic line of hybrid poplar expressing a *cry3Aa* gene, possibly caused by a higher mRNA turn-over or a lower rate of protein synthesis (Génissel et al. 2003).

To determine the copy number of the *cry8D2* transgene in the genome of the transgenic black ash lines, qPCR-based method was used. The relative standard curves for both the single-copy endogenous reference gene (*FnAG*) and *cry8D2* transgene were constructed using the serial dilution of genomic DNA (Fig. 3.6). Based on the values of slope (*S*) and intercept (*I*) from the curves, the following equations for *FnAG* and *cry8D2* were obtained: $Y_{FnAG} = -3.6635X + 30.092$ and $Y_{cry8D2} = -3.4842X + 31.331$, respectively. The coefficients of determination (R^2) were 0.9987 and 0.9998 for *FnAG* and *cry8D2*, respectively, indicating good reproducibility. Using these equations, the copy number of the *cry8D2* transgene was estimated, and the results revealed that all the transgenic lines contained two copies of the transgene (Table 3-3). In plant transformation studies, single- or low-copy insertions are desirable, as it generally yields stable transgene expression. However, random integration of transgenes via *Agrobacterium*-mediated transformation often produce multiple-copy insertions in various plant species (Abou-Alaiwi et al. 2012; Dong et al. 2001; Wang and Pijut 2014; Weng et al. 2004), that could result in transgene silencing through transcriptional and/or post-transcriptional gene silencing (TGS and PTGS), which were associated with DNA methylation in the promoter and the coding region, respectively (Fagard and Vaucheret 2000; Matzke et al. 1994). Higher degrees of cytosine methylation within

the GFP encoding region and CaMV 35S promoter region were found in transgenic sweet orange (*Citrus sinensis* L.) with multiple T-DNA copies when compared to the single copy transgenic plant (Fan et al. 2011). PTGS was observed in transgenic eastern white pine (*Pinus strobus* L.) with more than three copies of GFP transgene, whereas not in transgenic lines with one copy of T-DNA (Tang et al. 2007). In the present study, no gene silencing was observed in all three transgenic lines with two copies of *cry8D2* transgene (Fig. 3.5c).

3.5 Conclusions

In summary, an efficient *Agrobacterium*-mediated transformation system for black ash hypocotyls was established, and successfully applied to develop transgenic black ash shoots expressing the *Bt* insecticidal gene (*cry8D2*) for EAB resistance. Although transgenic *Bt*-black ash shoots showed growth retardation, strong expressions of mRNA and Cry8D2 toxin protein were confirmed. Studies to facilitate transgenic shoot elongation followed by adventitious root induction need to be established. Ultimately, a bioassay with EAB-adult and -larvae should be conducted to evaluate the efficacy of transgenic *Bt*-black ash shoots.

Table 3.1. Primer sequences.

Primer	Sequence (5' to 3')
cry_430_F	GAT CGC TCC TTG CGA CTA AC
cry_430_R	GCG TTG CAT AAC CAT TTG TG
nptII_F	TGC TCC TGC CGA GAA AGT AT
nptII_R	AGC CAA CGC TAT GTC CTG AT
cry_Full_F	GCA CAA TCC CAC TAT CCT TCG
cry_Full_R	ATT GCC AAA TGT TTG AAC GA
cry_qRT_F	TGG GAC TAT CAC GTC CAA CA
cry_qRT_R	GCG TAG TAG TTC GCG AGG TC
FnAG_qRT_F	CCT ATT ACT CTC TAC AAC TCG
FnAG_qRT_R	TGA GGA ATC TGA GCA GGC TTT C
eEFAα_F	ACC AGC AAG TCC CAG TTG AGA TG
eEFAα_R	TGA GCC AGG TTC AGC TTC CAA TG

Table 3.2. Effect of kanamycin concentration on percent callus formation and adventitious shoot regeneration of black ash hypocotyls.

Kanamycin (mg L^{-1})	Callus Formation[a] (%)	Shoot Induction[a] (%)
0	55.6 ± 2.8a	58.3 ± 8.3a
10	38.9 ± 2.8b	36.1 ± 2.8a
20	16.7 ± 4.8c	2.8 ± 2.8b
30	2.8 ± 2.8d	5.6 ± 2.8b
40	0d	0b
50	0d	0b

[a] Values represent means ± SE; followed by the same letter in the same column were not significantly different by the Duncan's multiple comparison test ($p < 0.05$).

Table 3.3. Estimated copy number of *cry8D2* transgene in transgenic black ash shoots determined by quantitative PCR (qPCR)-based method.

Sample	C_T (*FnAG*)	C_T (*cry8D2*)	2' (X_0/R_0)	Estimated copy number
T38	20.26 ± 00.2	22.92 ± 0.07	2.00 ± 0.16	2
T40-4	19.12 ± 0.10	21.31 ± 0.20	2.02 ± 0.37	2
T41-6	22.92 ± 0.06	24.31 ± 0.02	2.02 ± 0.29	2

Values are mean ± SD. *X0/R0* was calculated by using equation ($X0/R0 = 10[(C_{T,X} - I_X)/S_X] - [(C_{T,R} - I_R)/S_R]$. From standard curves of *FnAG* and *cry8D2*, the values of slope (*S*) and intercept (*I*) were obtained. Each qPCR reaction was repeated three times.

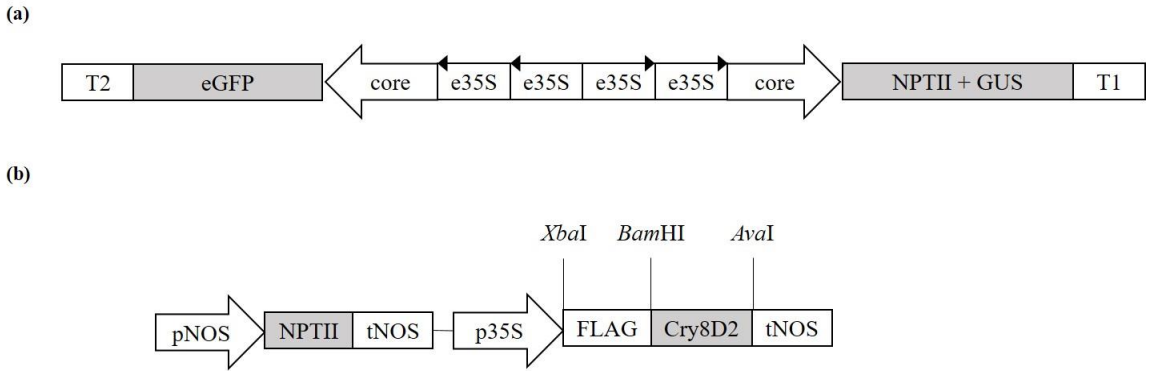

Figure 3.1. Schematic diagram of T-DNA regions of pq35GR and pBI121-*cry8D2* constructs. (a) The pq35GR vector consisted of bi-directional CaMV 35S promoters containing two divergently arranged enhancer repeats, an *eGFP* gene, and an *NPTII* and *GUS* fusion gene (Li et al. 2004). eGFP, enhanced green fluorescent protein gene; core, 35S core promoter; e35S, enhancer fragment isolated from the CaMV 35S promoter; NPTII + GUS, neomycin phosphotransferase gene and β-glucuronidase gene; T1 and T2, terminator and polyadenylation signal sequences from the NOS gene and the 35S transcript, respectively; (b) A binary vector pBI121 was used for insertion of synthetic *cry8D2* gene driven by CaMV 35S promoter. pNOS and tNOS, promoter and terminator sequence from the NOS gene, respectively.

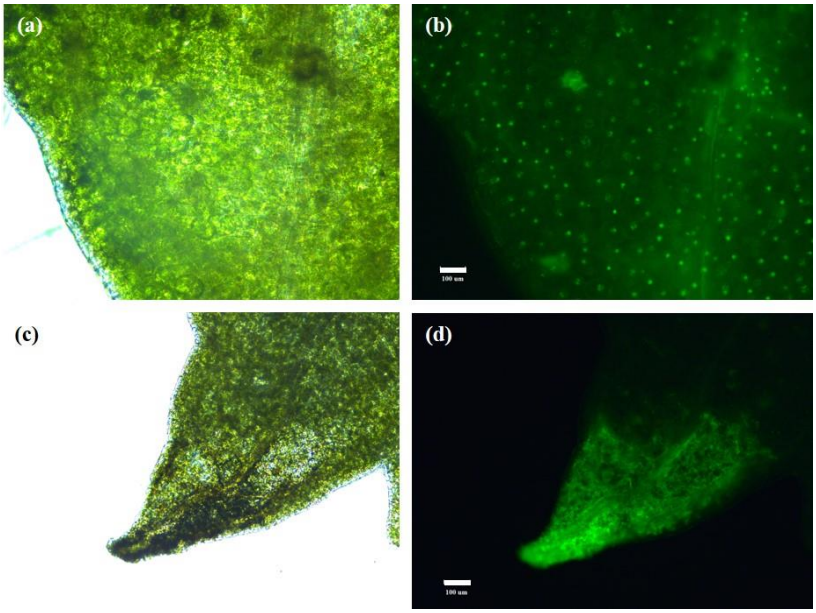

Figure 3.2. Visualization of green fluorescent protein (GFP) from transformed leaves under bright field image (a and c) and through GFP excitation filter (470/40 nm) (b and d) (*bar* = 100 µm).

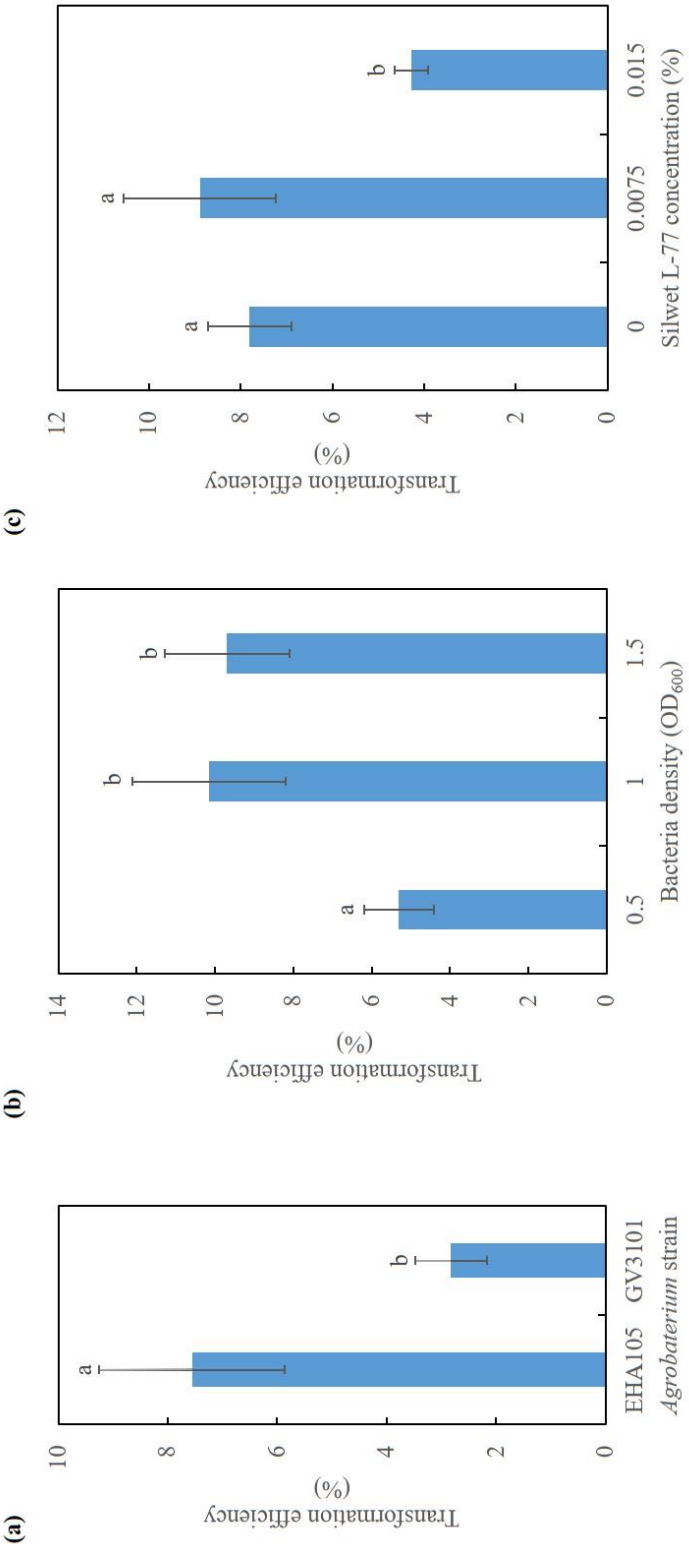

Figure 3.3. *Agrobacterium*-mediated transformation efficiency in black ash hypocotyls. Effects of (a) *Agrobacterium* strain, (b) bacteria density, and (c) Silwet L-77 concentration on transformation. Values are means ± SE for 40 to 52 explants per treatment, replicated three times. Different letter indicates a significant difference based on a Duncan's multiple comparison test ($p < 0.05$).

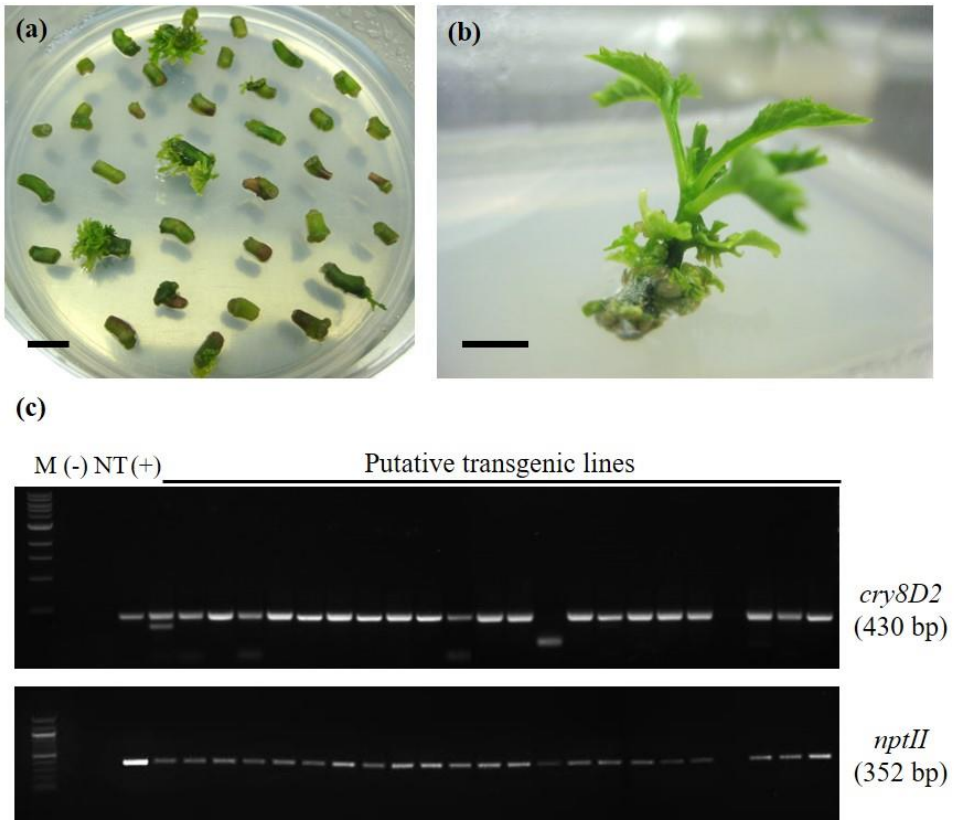

Figure 3.4. *Agrobacterium*-mediated transformation and regeneration of transgenic black ash expressing *cry8D2*. (a) *In vitro* selection of transformed hypocotyls on medium containing 40 mg L^{-1} kanamycin (*bar* = 1 cm); (b) Adventitious shoot regenerated from putative transgenic hypocotyl (*bar* = 1 cm); (c) Detection of a 430-bp fragment of *cry8D2* transgene and a 352-bp fragment of *nptII* via PCR from the leaves of putative transgenic shoots. M, DNA ladder (1-kb for *cry8D2* and 100-bp for *nptII*); (–), water control; NT, negative control of non-transformed black ash DNA; (+), positive vector control.

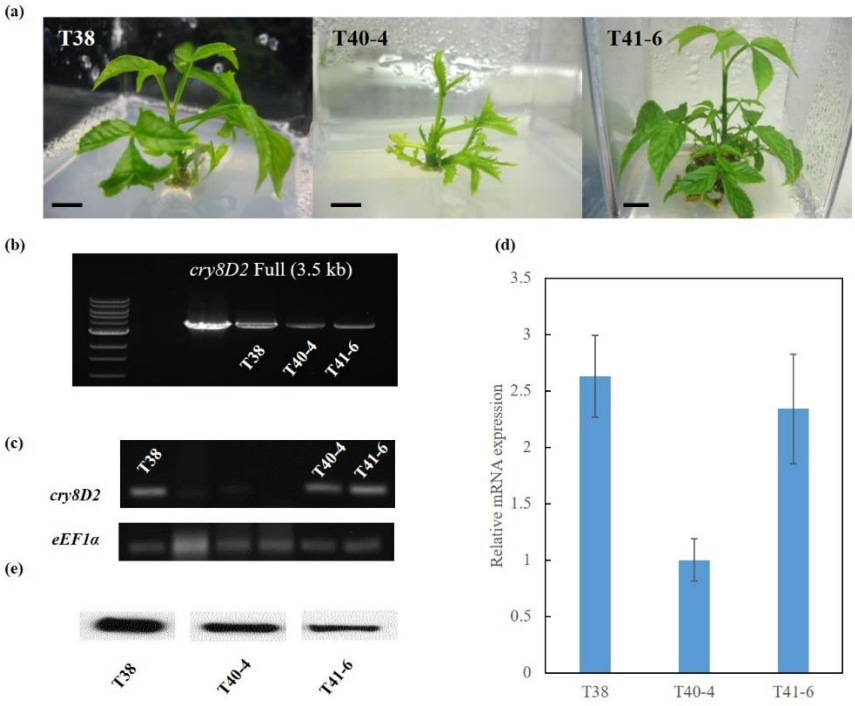

Figure 3.5. Confirmation of the insertion of a full-length *cry8D2* transgene, *cry8D2* transcript expression, and Cry8D2 protein accumulation in transgenic black ash shoots. (a) Three independent transgenic black ash shoots harboring *cry8D2* gene (*bar* = 1 cm); (b) Detection of a 3.5-kb full-length *cry8D2* transgene via PCR from the leaves of transgenic shoots. Lane 1, 1-kb DNA ladder; lane 2, negative control of non-transformed black ash DNA; lane 3, positive vector control; lanes 4-6, three independent transgenic lines; (c) Semi-quantitative RT-PCR analysis and (d) real time qPCR for detecting *cry8D2* transcript expression in the leaves of transgenic shoots. The translation elongation factor alpha (*eEF1α*) gene was used as a control to normalize the expression level. Each reaction was repeated three times. Error bars represent the standard deviation; (e) Western blot analysis to detect Cry8D2 protein expression in the leaves of transgenic shoots. A monoclonal anti-FLAG antibody was used. The Cry8D2 molecular weight observed in the Western blots was about 130 kDa.

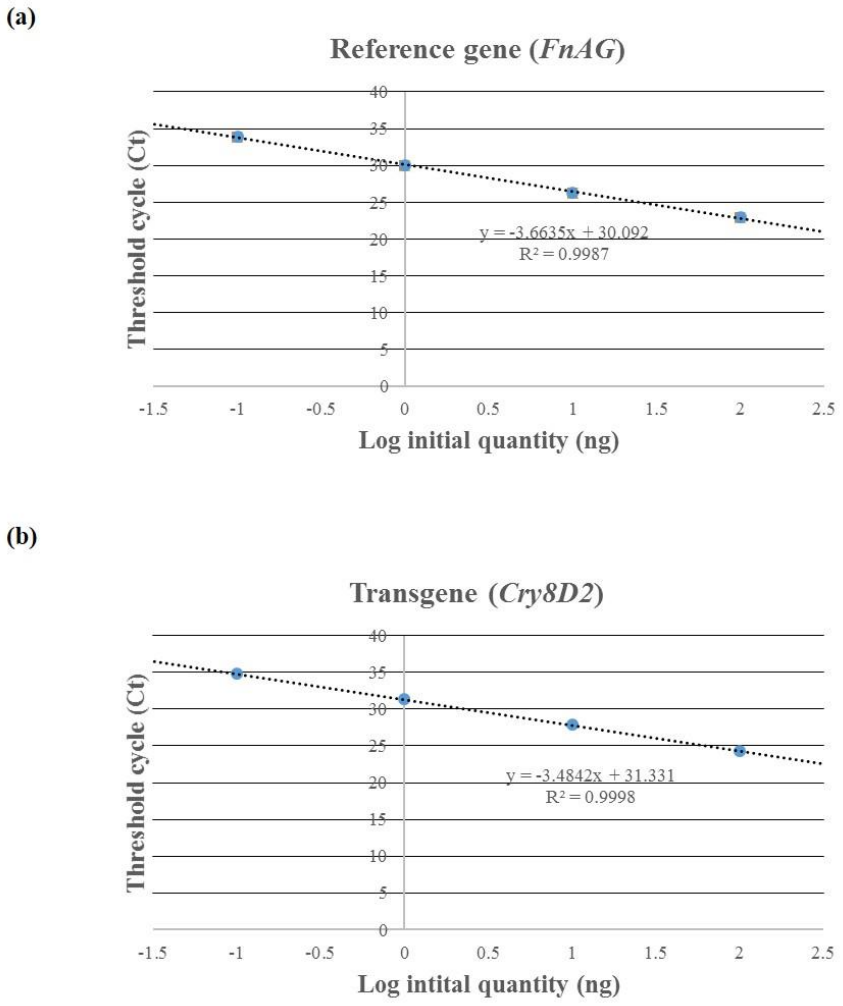

Figure 3.6. The relative standard curve of (a) *FnAG*, a single-copy endogenous reference gene and (b) *cry8D2* transgene, obtained by plotting the threshold cycle (C_T) value versus the log of each initial quantity of genomic DNA.

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CHAPTER 4. ISOLATION AND CHARACTERIZATION OF A FLORAL HOMEOTIC GENE IN *FRAXINUS NIGRA* CAUSING EARLIER FLOWERING AND HOMEOTIC ALTERATIONS IN TRANSGENIC *ARABIDOPSIS*

4.1 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.

Keywords: AGAMOUS, black ash, ectopic expression, flowering, *Fraxinus nigra*, MADS.

4.2 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 el 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 (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).

4.3 Materials and Methods

4.3.1 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.

4.3.2 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 4.1) 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 transcription 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) (FirstChoice RLM-RACE; Life Technologies, Grand Island, NY, USA). For the first-round PCR, AGP3 and AGP4 (Table 4.1) were used as 5´- and 3´-RACE outer primers, respectively. For the second-round PCR, AGP5 and AGP6 (Table 4.1) 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 4.1) 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*.

4.3.3 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 1,000 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 (X53579; *A. thaliana*), JcAG (NP_001292936; *Jatropha curcas*), PtAG1 (AF052570; *Populus trichocarpa*), PtAG2 (AF052571; *P. hybrida*), TcAG (XP_007025251; *Theobroma cacao*), PsAG (EU938540.1; *P. serotina*), PpMADS4 (AAU29513; *P. persica*), PmAG (ABU41518; *P. mume*), PLE (BAI68391; *Antirrhinum majus*), AGL1/SHP1 (AEE79831.1; *A. thaliana*), AGL5/SHP2 (AEC10175.1; *A. thaliana*), FBP11 (CAA57445.1; *Petunia hybrida*), VvMADS5 (AAM21345.1; *V. vitis*), MdMADS10 (NP_001280931.1; *Malus domestica*), AGL11/STK (AEE82819.1; *A. thaliana*), CAG1 (NP_001267506.1; *Cucumis sativus*), PhaAG2 (AAS45706.1; *Phytolacca americana*), and SxcAG2 (AAS45704.1; *Micranthes careyana*).

4.3.4 Expression analysis of *FnAG* in black ash

To analyze the relative expression of *FnAG*, semi-quantitative RT-PCR and real-time PCR (qPCR) were performed. Total RNAs were extracted from vegetative tissues (leaves and in vitro shoot cultures) and reproductive tissues (male and female flowers) of black ash using the RNeasy Plant Mini Kit (Qiagen). DNase I (Thermo Fisher Scientific) treated RNA was then used in RT-PCR to synthesize first-strand cDNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and an oligo-dT primer. The qPCR was performed with 20 µl reaction solution containing 1 µl cDNA, 10 µM AGP3 and AGP6, and iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). The cycling conditions consisted of DNA polymerase activation at 95°C for 60 s, 40 cycles of 95°C for 10 s, 57°C for 20 s, 72°C for 30 s, and followed by a melting curve analysis from 65 to 95°C performed with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Relative transcript levels for mRNAs were obtained using the comparative cycle threshold (C_t) method and normalized to translation elongation factor alpha (*eEFAα*) from black ash (Rivera-Vega et al. 2012). Each reaction had three biological replicates and was repeated twice.

4.3.5 Functional analysis of *FnAG* through transformation of *Arabidopsis thaliana*

A binary vector pBI121 was used for *FnAG* insertion and overexpression in *Arabidopsis*. The GUS gene in pBI121 was replaced by the CDS sequence of *FnAG* which was driven by the CaMV 35S promoter. The *35S::FnAG* construct and the empty pBI121 vector as a control were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation (Mattanovich et al. 1989), and then introduced into the wild-type *A. thaliana* ecotype Col-0 via the floral dip method (Zhang et al. 2006). T_1 seeds were placed onto agar plates containing MS medium with 50 mg L^{-1} kanamycin as selection agent. The seedlings were screened for 2-3 weeks on the plate and then kanamycin-resistant lines were transferred to water-saturated soil in pots. The integration of *FnAG* in the transgenic lines was confirmed by PCR using AGP3 and AGP4 primers. The number of days from planting to the first flower and the number of rosette leaves at that stage were recorded in T_3 plants. Statistical differences were assessed via analysis of variance with the SAS® 9.3 software package (SAS® Institute 2011).

4.3.6 Expression analysis of *AtAG* and *FnAG* in transgenic *Arabidopsis*

Floral tissues were collected from seven transgenic lines and wild-type *Arabidopsis*. The qPCR was performed as previously described. Primers, AtAG1 and AtAG2 (Table 4.1) were designed to amplify endogenous *AG*, and AGP3 and AGP6 were used to amplify ectopically expressed *FnAG*. Relative transcript levels for mRNAs were obtained using the C_t method and normalized to *ACTIN2* from *Arabidopsis* (AtActin1 and AtActin2 as forward and reverse primers, respectively). Each reaction had three biological replicates and was repeated twice.

4.4 Results

4.4.1 Isolation and sequence analysis of an *AG* ortholog from black ash

In order to isolate the *AG* ortholog from black ash, RT-PCR was performed using degenerate primers developed based on highly conserved sequence from a total of 17 *AG* homologs. An expected 258-bp internal fragment was amplified and sequenced (data not shown) to design specific primers for RACE. The results of 5´- and 3´-RACE revealed that *FnAG* cDNA was 1022-bp in length with a 5´ untranslated region (UTR) of 67-bp and a 3´ UTR of 226-bp upstream of the poly(A) tail. A 4578-bp genomic sequence was obtained and the alignment analysis of cDNA and genomic sequence showed that *FnAG* consisted of nine exons and eight introns (Fig. 4.1a). *FnAG* encodes a putative type II plant MADS-box protein of 242 amino acids containing N-, MADS-, I-, K-, and C-domains. It also contains two short, highly conserved regions, called AG motif I and AG motif II in the C-domain, as reported for other AG homologs (Kramer et al. 2004). A comparison of deduced amino acid sequence of *FnAG* and *AG* homologs from other woody species showed a highly conserved 56 residue MADS-domain (Fig. 4.1b). FnAG shared greater than 96% amino acid identity to the other AG homologs within the MADS-domain. In addition to the MADS-domain, regions including I- and K-domains were also conserved among FnAG and other AG homologs (Fig. 4.1b). Overall, FnAG was most similar to FpAG from *F. pennsylvanica*, showing 94% identity, followed by 81% identity with GAG2 from *Panax ginseng*, 77% with PtAG2 from *Populus trichocarpa*, 75% with PsAG from *Prunus serotina* and PtAG1, 74% with PpMADS4 from *Prunus persica*, and 68% with AG from *Arabidopsis thaliana* and MdAG from *Malus domestica*. A phylogenetic tree was constructed using the deduced amino acid sequence of *FnAG* and 28 other MADS-box proteins (Fig. 4.2). Phylogenetic analysis placed *FnAG* within the

clade of C-lineage *AG* subfamily genes, showing high bootstrap support for a close relationship with SiAG from *Sesamum indicum* and CsAG from *Citrus sinensis*.

4.4.2 Expression analysis of *FnAG* in black ash tissues

The spatial expression of *FnAG* was investigated in various vegetative and reproductive tissues using semi-quantitative RT-PCR (Fig. 4.3a). Gene-specific primers were used to amplify the 183-bp long internal fragment of *FnAG*. Amplification of an *eEFAα* fragment was used as a constitutively expressed gene control to enable inter-sample comparisons. The transcripts of *FnAG* were detected in the female and male flowers and in vitro shoot cultures, but only rarely in leaves. The steady state levels of *FnAG* in reproductive tissues were higher than in in-vitro shoot cultures, showing a higher relative intensity of amplified product. The relative mRNA expression level of *FnAG* was also examined by qPCR (Fig. 4.3b). The mRNA levels of *FnAG* were 25.8- and 13.5-fold higher in female and male flowers, respectively, than in leaves. Its level in in-vitro shoot cultures was 2.3-fold higher than in leaves.

4.4.3 Ectopic expression of *FnAG* in *Arabidopsis*

We used ectopic expression of *FnAG* CDS in *Arabidopsis* to determine whether *FnAG* could function as *AG*. A binary vector pBI121 containing *35S*::*FnAG* and neomycin phosphotransferase II (*nptII*) gene was introduced into *Arabidopsis* ecotype Col-0 via *Agrobacterium* transformation. Among a total of 17 kanamycin-resistant T_1 plants, the presence of *FnAG* was confirmed from seven independent plants (data not shown) and these were used to generate T_3 plants for phenotyping. Phenotypic alterations were observed in *FnAG*-overexpressing plants (Fig. 4.4). The most prominent changes in the *FnAG*-overexpressing transgenic plants were the homeotic modifications in the first and second whorls of flowers. Compared to wild-type flowers consisting of four sepals, four petals, six stamens, and a pistil (Fig. 4.4a), transgenic flowers displayed petals transformed into stamen-like structures (Fig. 4.4b), and sepals converted into carpel-like structures (Fig. 4.4c). Third and fourth whorls developed normally, resulting in fertility. The *35S::FnAG* plants also showed small and curled leaves (Fig. 4.4d), immature early flowers with sepals which failed to enclose flower buds (Fig. 4.4e), and reduced plant size (Fig. 4.4f). *FnAG*-overexpressing plants flowered significantly earlier than wild-type and empty-vector control plants (Fig. 4.4g-i).

Based on the flowering time and phenotypic alteration, transgenic plants were classified into two groups (Fig. 4.5). Although plants with weak *ap2*-like phenotype (Fig. 4.5b) showed relatively normal vegetative growth compared to strong phenotype plants (Fig. 4.5a), they displayed smaller plant size than wild-type plants (Fig. 4.5c). Plants with strong phenotype produced first flowers 12.62 days earlier (with 5.19 rosette leaves) than the wild-type, while plants with weak phenotypic alteration produced first flowers 6.83 days earlier (with 10.1 rosette leaves) than the wild-type (Table 4.2). The *FnAG* mRNA expression level was higher in the strong phenotype group (#1, #3, #4, and #6) than that in the weak phenotype group (#2, #5, and #7) (Fig. 4.6a). The endogenous *AtAG* mRNA expression, however, was not significantly different among plants in all groups (Fig. 4.6b).

4.5 Discussion

During the diversification of the *AG* subfamily genes in angiosperms, gene duplication and functional evolution have occurred, giving rise to the C- and D-lineage (Kramer et al. 2004). Thus, the D-lineage genes such as *SEEDSTICK* (*STK*; formerly known as *AGL11*) also belong to the *AG* subfamily and were preferentially recruited for ovule development (Dreni and Kater 2014). Subsequent duplications in the C-lineage have led to paralogous lineages of *PLENA* (*PLE*) from *Antirrhinum* and eu*AG* from *Arabidopsis*, maintaining functional redundancy (Kramer et al. 2004). Although *PLE* C-lineage genes in *Arabidopsis*, such as *SHATTERPROOF1* (*SHP1*) and *SHP2* (formerly known as *AGL1* and *AGL5*, respectively), are similar to *AG* in function, they were not expressed in the meristem and primordial cells at the appropriate time, so *AG* was the only fully functional C-class gene (Dreni and Kater 2014). In the present study, phylogenetic analysis confirmed that *FnAG* was placed within the eu*AG* C-lineage clade, indicating that *FnAG* was associated with C-function of *AG*.

FnAG showed typical gene structure; the position and number of introns in *FnAG* was conserved with respect to other *AG* homologs (Brunner et al. 2000; Kramer et al. 2004). Unlike other MIKC-type MADS-box genes that have six introns, genes in the C-lineage of *AG* subfamily including *FnAG* have two additional introns positioned 5′ of the MADS domain and in the last codon of AG motif II (Kramer et al. 2004). In *FnAG*, the second intron was about 2 kb spanning the largest part of the gene, which might contain functionally important regulatory sequences for controlling its expression specifically to stamens and carpels. Previous studies in *Arabidopsis*

demonstrated that the second intron contains cis-regulatory elements that are the binding sites of activators such as LEAFY and WUSCHEL, or repressors such as AP2 or LEUNIG, to control *AG* expression (Sieburth and Meyerowitz 1997; Deyholos and Sieburth 2000; Hong et al. 2003). This binding motif was also highly conserved in poplar (Brunner el al. 2000). It has been suggested that using the enhancer element in the second intron of *AG* could produce complete sterility in *Arabidopsis* and tobacco through tissue-specific ablation of stamens and carpels (Liu and Liu 2008; Wang et al. 2008). Although we did not study this regulatory element in detail, this approach would likely be a practical method to achieve gene containment in transgenic trees.

A previous study revealed that there was only one *AG* homolog in green ash (Du and Pijut 2010). A BLAST search of *FnAG* against the European ash (*F. excelsior*) genome (www.ashgenome.org) showed that European ash may contain two *AG* homologs (personal communication with Richard Buggs, Queen Mary University of London).

The deduced amino acid sequence of FnAG revealed extensive sequence similarity with other AG proteins. As a major determinant of DNA binding, the MADS-domain was highly conserved in FnAG. As was found for AG and proteins of AG homologs, FnAG included an additional peptide extension of 17 amino acid residues at the N-terminal before the MADS domain (Fig. 4.1b). The N-terminal extension peptide, however, does not appear to affect AG activity, because proteins of some AG homologs that lack this extension, such as LLAG1 in lily, HpAG in *Hosta plantaginea*, and CiAG in pecan, function normally (Mizukami et al. 1996; Benedito et al. 2004; Wang et al. 2012, Zhang et al. 2016). The I- and K-domains of *FnAG* were moderately conserved, but showed high sequence similarity to those of *FpAG*. According to Pnueli et al. (1994), in this region, tomato showed a closer relationship with tobacco than Brassicaceae, and *Antirrhinum* was more closely related to the Solanaceae than Brassicaceae, suggesting the apparent evolutionary relationship between species in this region.

The C-domain is the most variable region and its function has not yet been elucidated. Studies of truncated AG protein lacking the C-domain, however, showed *ag*-like phenotype, indicating that the truncated AG protein inhibits normal AG function (Mizukami et al. 1996). Complete male sterility was obtained only in transgenic tobacco ectopically expressing truncated PrAG1 protein without the K- and C-domain, but not in lines expressing the full-length protein, indicating that the C domain or C plus K domains may affect the MADS dimer formation between PrAG1 and NAG1 (Liu 2012). In *Prunus lannesiana*, a 170-bp exon skip caused by abnormal

splicing deleted the C-domain AG motifs I and II. The result was a double-flowered phenotype in which stamens and carpels were converted to petaloid organs (Liu et al. 2013). Overall, the C-domain is functionally important, and it could be involved in transcriptional activation or higher-order protein interactions for the formation of multimeric transcription factor complexes (Theissen et al. 2000; Kramer et al. 2004).

In addition to evidence from amino acid sequence analyses of FnAG, the steady state transcript pattern of *FnAG* strongly indicated that *FnAG* was the ortholog of *AG*. The spatial pattern of transcript abundance for *FnAG*, specifically its abundance in reproductive tissues, corresponded with that of *Arabidopsis AG* and other *AG* homologous genes (Yanofsky et al. 1990; Du and Pijut 2010; Liu 2012; Zhang et al. 2013). Although *FnAG* transcripts were also detected in vegetative tissues, as observed in poplar (Brunner et al. 2000) and pecan (Zhang et al. 2016), the steady state levels in vegetative tissue were significantly lower than in reproductive tissues.

In nature, double-flowered mutants provide strong evidence that homeotic mutant phenotypes of this class derive from mutations in *AG* homologous genes. A study with a double-flowered ranunculid mutant 'Double White' revealed that the insertion of a retrotransposon within the putative *AG* ortholog *ThtAG1* caused either nonsense-mediated decay of transcripts or alternative splicing that resulted in mutant proteins with K-domain deletions (Galimba et al. 2012). Loss of C-domain AG motifs I and II in *PreAG* caused by abnormal splicing produced the double-flowered cultivar of *Prunus lannesiana* (Liu et al. 2013). Even though there were no significant differences between the double-flowered cultivar and wild-type plants in promoter and intron sequences, the level of *TrimAG* transcript was reduced in the double-flowered *Tricyrtis macranthopsis* cultivar, indicating it might derive from a mutation in one of the genes in the regulatory network that controls *TrimAG* expression or, alternatively, transcriptional silencing by methylation of *TrimAG* promoter/intron sequences (Sharifi et al. 2015).

The function of isolated *AG* homologs usually has been examined by ectopic expression in model plants to see if the transgenic plants showed homeotic alteration. That was because complementation analysis was more difficult, as the null-mutant of *AG* was completely sterile. However, not all *AG* homologous genes cause the typical homeotic alteration of *ap2*-like phenotype in transgenic plants when ectopically overexpressed. The ectopic expression of *NTAG1* from Chinese narcissus rarely produced the homeotic floral phenotype, especially as the generation of transgenic plants increased (Deng et al. 2011). Liu (2012) also found no phenotypic effect on

floral development from transgenic tobacco plants with ectopic expression of *PrAG1*. This could be because there were different interacting cofactors in ectopic tissues, or endogenous genes in transformed plants masked the functionality of *AG* homologs from other species (Liu 2012). The long juvenility of black ash (10-15 years) significantly complicates transgenic evaluation of flowering gene action within the species. Therefore, in the present study, we used *Arabidopsis* for the functional analysis of *FnAG*. The ectopic expression of *FnAG* in transgenic *Arabidopsis* induced homeotic conversions of carpeloid sepals and stamenoid petals, curled leaves, reduced plant size, and prematurely terminated inflorescences, as has been reported for other *AG* homologs (Du and Pijut 2010; Wang et al. 2012; Liu et al. 2013). The transgenic plants also showed accelerated flowering: 12.62 and 6.83 days earlier in strong and weak phenotypes, respectively, than wild-type. Ectopic expression of the Gm*GAL2*, a soybean *AGAMOUS Like 2*, enhanced flowering in transgenic *Arabidopsis* regardless of the photoperiod by promoting the expression of key flowering genes *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*), and suppressing floral inhibitor *FLOWERING LOCUS C* (*FLC*) (Xu et al. 2010).

4.6 Conclusions

In summary, an *AG* ortholog was cloned from black ash and its function in floral organ identity was analyzed. Sequence analysis and expression analysis showed that *FnAG* was closely related to the other *AG* homologs with transcript expression specifically in the reproductive tissues. *FnAG* was involved in floral organ identity, as expected for a functional homolog of *AG* in black ash. Based on these data, we conclude that *FnAG* is a strong candidate for the black ash functional ortholog of *Arabidopsis AG*, and we believe *FnAG* will be an excellent target for genome editing to produce transgenic sterile black ash. Our goal is to manipulate *FnAG* using a targeted genome modification technique -clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system- for production of sterile black ash. CRISPR/Cas9 constructs targeting *FnAG* are being developed and transformation is currently underway.

Table 4.1. 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

Table 4.2. Flowering times of wild-type and transgenic *Arabidopsis*.

Genotype	Days to flowering	No. leaves	No. plants
Wild-type (Col-0)	32.79 ± 0.83	14.89 ± 0.78	9
35S::FnAG weak	25.96 ± 1.40	10.10 ± 1.07	27
35S::FnAG strong	20.17 ± 1.44	5.19 ± 1.51	36

Values represent means ± SD.

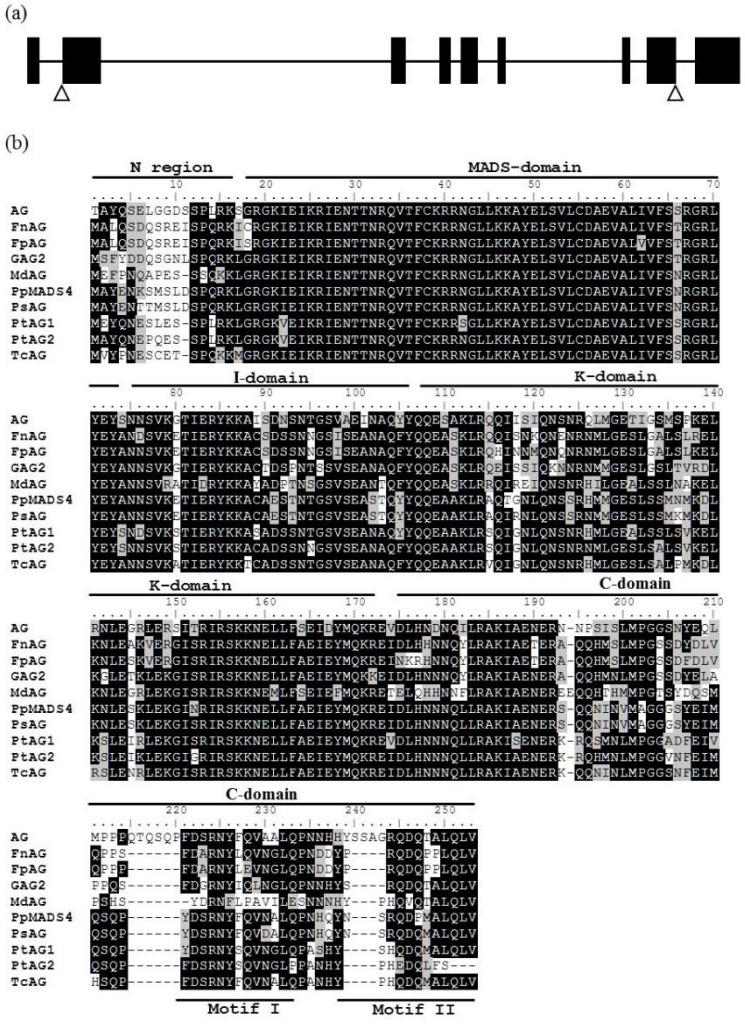

Figure 4.1. Gene structure and deduced amino acid sequence alignment of an *AGAMOUS* ortholog in black ash (*FnAG*). (a) *FnAG* gene structure. Exons are depicted as *black boxes* and introns by *lines*. The *triangles* represent the position of start and stop codons. (b) Alignment of deduced amino acid sequences encoded by *FnAG* with *AG* homologs from a range of woody plant species. Identical amino acid residues in relation to FnAG are *black* and conserved residues are in *gray*. *Dots* indicate gaps inserted for alignment optimization. The amino acid terminal extension (N), MADS, intervening (I), keratin-like (K), and carboxyl terminal (C) regions are marked. Motifs I and II within the C region were indicated.

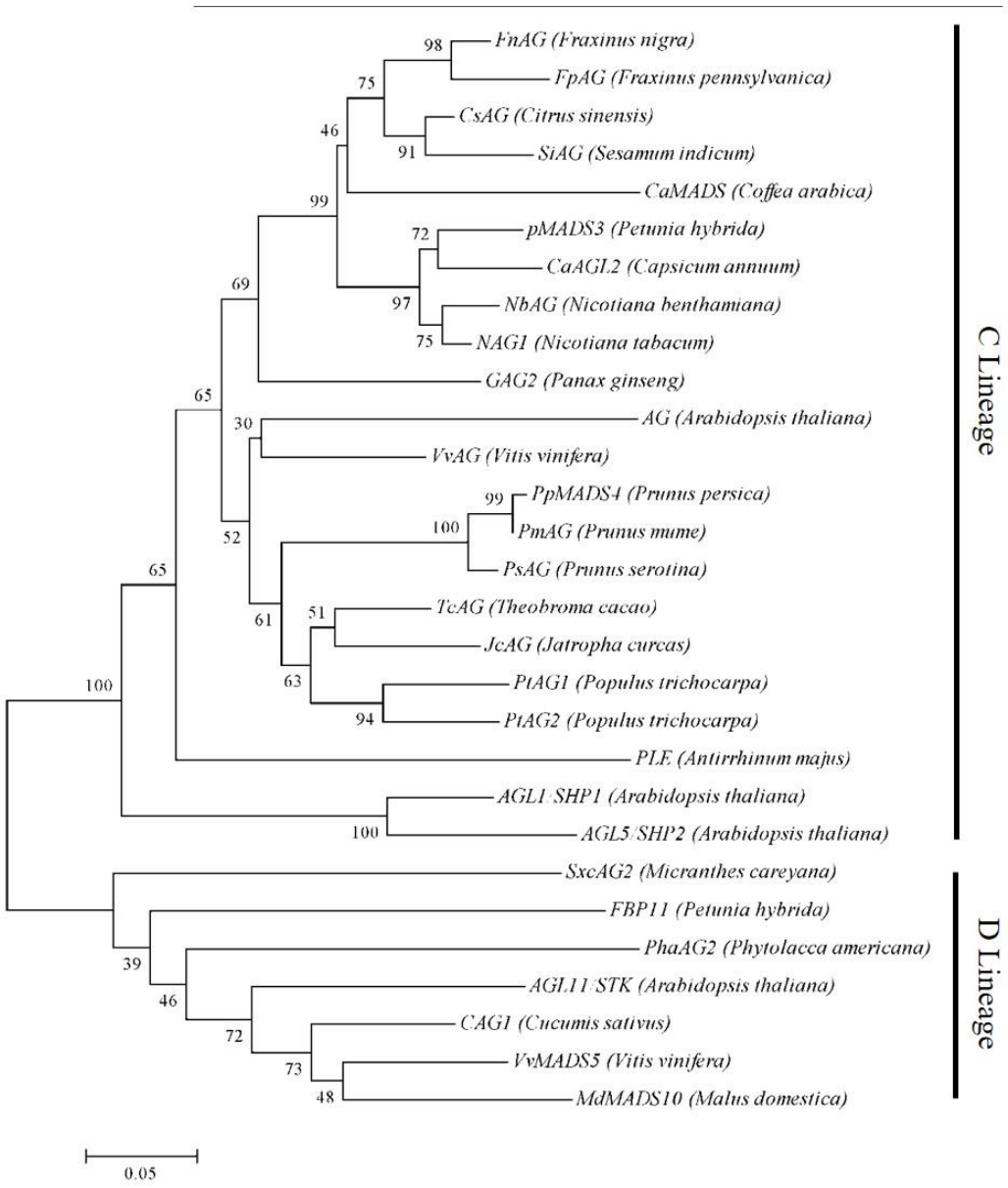

Figure 4.2. Phylogenetic analysis of MADS-box proteins by the neighbor-joining method. The bootstrap confidence values (%) from 1000 replicates are indicated on the *branches*. The *scale at the bottom* indicates genetic distance proportional to the amino acid substitutions per site.

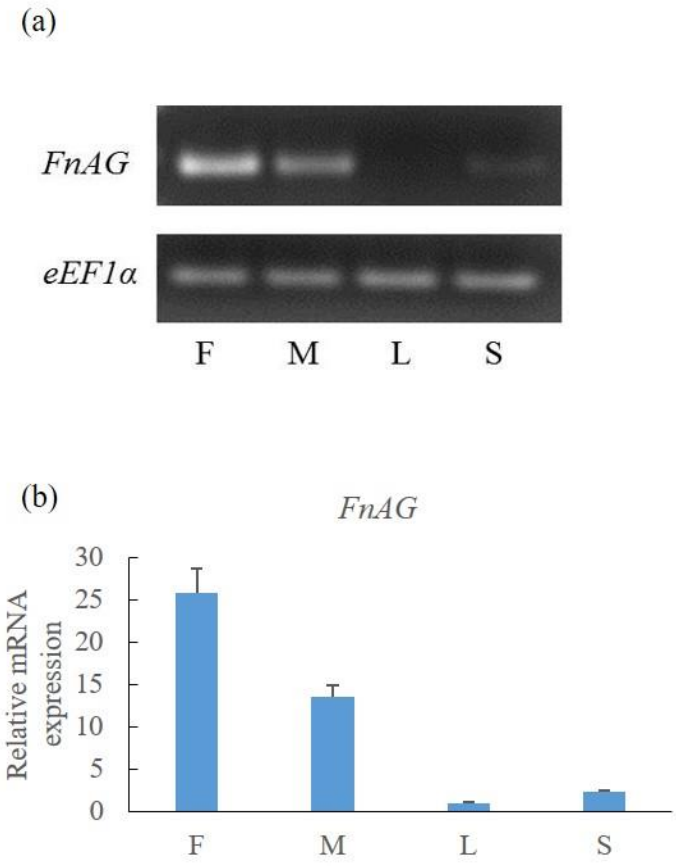

Figure 4.3. Expression pattern of *FnAG* in various tissues of black ash by (a) Semi-quantitative RT-PCR analysis and (b) real-time PCR analysis. F, female flowers; M, male flowers; L, leaves; and S, in-vitro shoot cultures. The translation elongation factor alpha (*eEF1α*) gene was used as a constitutively expressed control. Each reaction had three biological replicates and was repeated two times. Error bars represent the standard deviation.

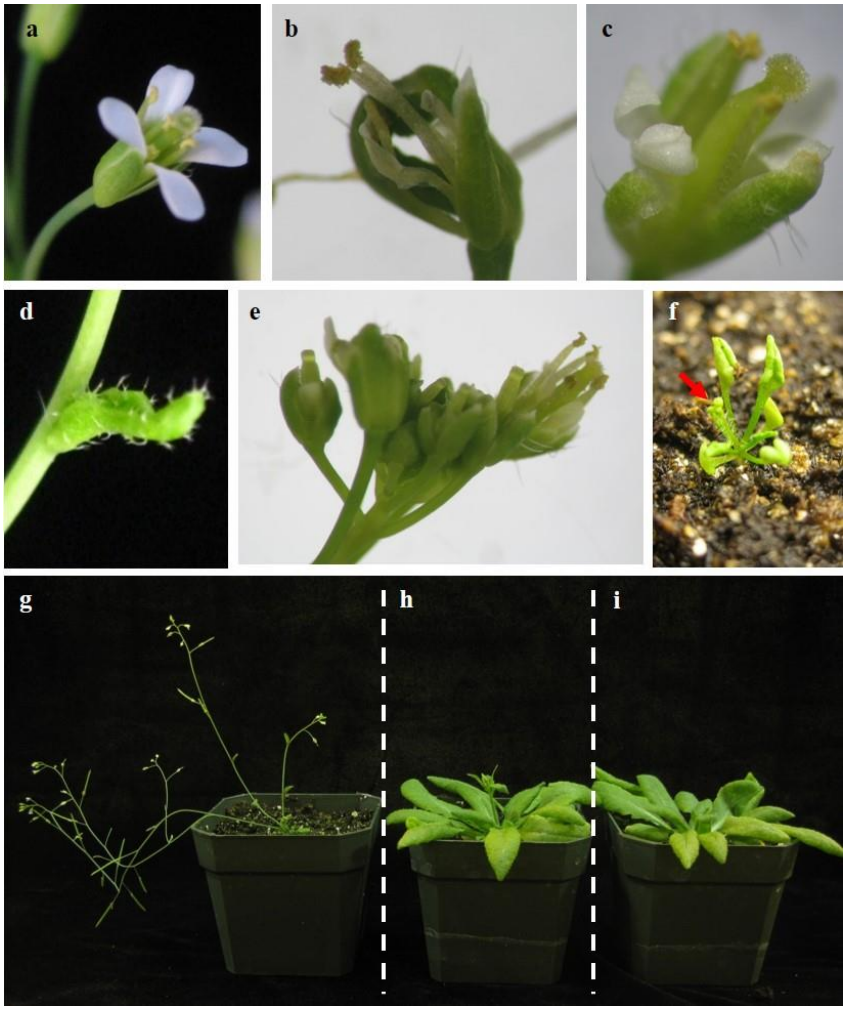

Figure 4.4. Floral and vegetative morphology of *Arabidopsis*. (a) Wild-type flower. Transgenic flowers overexpressing *FnAG* under the 35S promoter showed homeotic mutations in the first and second whorls including (b) stamen-like petals, or (c) sepals converted into carpel-like structures. (d) Curled transgenic cauline leaf entrapping the lateral inflorescence. (e) Immature early flowers showing failure of the sepals to enclose flower buds. (f) Extremely small size transgenic plant with early bolting. Arrow indicates floral buds. Thirty-one-day-old (g) transgenic, (h) wild-type, and (i) empty vector control plants.

Figure 4.5. Phenotypes of transgenic *Arabidopsis* (*35S::FnAG*) plants with (a) strong or (b) weak phenotypic alterations compared to (c) wild-type plant.

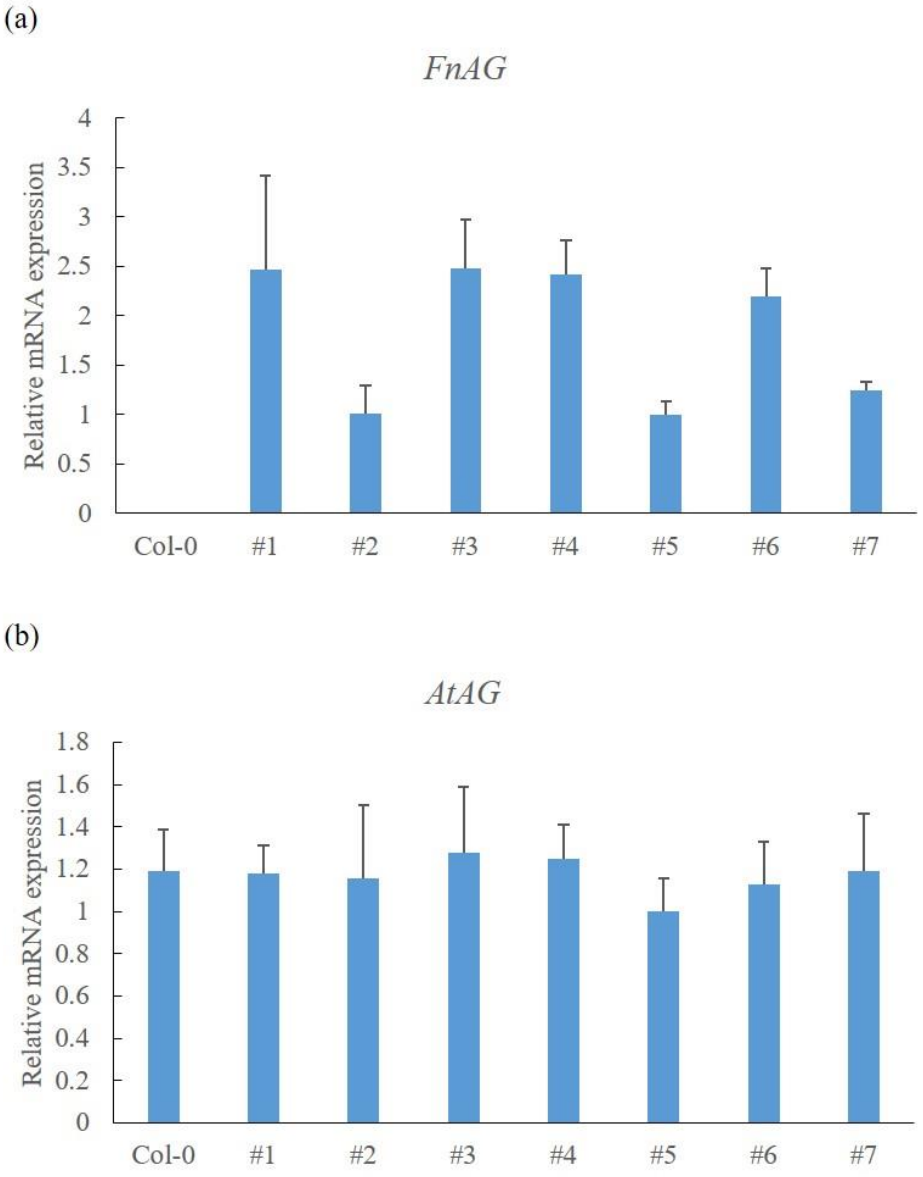

Figure 4.6. Relative expression levels of (a) ectopically expressed *FnAG* and (b) endogenous *AtAG* in wild-type (Col-0) and transgenic *Arabidopsis*. Expression levels were normalized to *AtActin2*. Each reaction had three biological replicates and was repeated twice. Error bars represent the standard deviation.

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CHAPTER 5. TARGETED GENOME EDITING OF *FRAXINUS NIGRA* FOR REPRODUCTIVE STERILITY USING THE CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR)/CRISPR-ASSOCIATED PROTEIN9 (CAS9) SYSTEM

5.1 Abstract

Transgene flow from genetically modified (GM) trees is a major concern. It is the main reason for stringent regulations and low public-acceptance of GM trees. To achieve gene containment, reproductive sterility can be obtained by disrupting genes involved in development of floral organs. I tested a total of five clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (Cas9) constructs to induce mutations at three target sites within an *AGAMOUS* ortholog of *Fraxinus nigra* (*FnAG*) that is responsible for stamen and carpel development. Using *Agrobacterium*-mediated transformation, transgenic black ash shoots harboring CRISPR/Cas9 vectors were produced. The transformation frequency was 0.82%, but the mutation rate based upon a total of 6,069 inoculated explants was 0.03%. Among 6,069 explants, 50 independent lines were confirmed to contain an integrated *Cas9* expression cassette. Of these, only two showed mutations in the target site. Both were one nucleotide substitution at two base pairs upstream from the protospacer adjacent motif. *Cas9* transgene silencing was observed from some transgenic lines that showed no mutations, indicating that more efficient promoters are required for stable *Cas9* expression in black ash, and these may be key to improved genome editing efficiency.

Keywords: *AGAMOUS*, black ash, CRISPR/Cas9, *FnAG*, gene containment, genome editing, reproductive sterility.

5.2 Introduction

In recent decades, biotechnological advancements in genetic engineering have led to a great impact not only on modern agriculture through crop improvement, but also in the forestry sector. Genetic modification technology has been applied to forest tree species to impart pest- or herbicide-resistance (Grace et al. 2005; Harcourt et al. 2000; Klocko et al. 2013; Lachance el al. 2007; Li et al. 2008), to improve wood quality related to biomass yield or lignin content and composition (Leplé et al. 2007, Plasencia et al. 2016; Unda et al. 2017; Voelker et al. 2010), and to improve abiotic stress-tolerance (Li et al. 2009; Matsunaga et al. 2012; Yu et al. 2013). The most important application of genetic modification technology would be fundamental research on forest tree biology to elucidate gene function or regulatory mechanisms. Despite its substantial benefits, stringent regulations and low public-acceptance of genetically modified (GM) forest trees limits research progress and commercial applications (Elorriaga et al. 2014). The risk of potential transgene flow through pollen dispersal, a process of transgene movement to wild- or weedy-relatives that potentially leads to hybridization or introgression and its environmental impacts, are the major controversial issue.

In order to alleviate these concerns, several transgene containment strategies have been discussed (Brunner et al. 2007; Daniell 2002), and reproductive sterility was the most extensively studied approach. In a few forest tree species, attempts have been made to destroy tissues essential for gamete development by using floral tissue-specific promoters. One such promoters, TA29 tapetum-dominant promoter, fused to a cytotoxin gene such as *BARNASE*, resulted in male sterility (Elorriaga et al. 2014; Höfig et al. 2006; Zhang et al. 2012). Growth retardation and vegetative abnormality, however, were reported in *BARNASE* transgenic poplar and birch, probably because low levels of BARNASE were expressed and caused cytotoxicity in vegetative tissues (Elorriaga et al. 2014; Lännenpää et al. 2005; Wei et al. 2007). RNA interference (RNAi) was developed to suppress genes involved in floral initiation and floral organ identity, such as *AGAMOUS* (*AG*) (Klocko et al. 2016). *AG* is a C-class floral homeotic gene responsible for stamen and carpel development. As mutant flowers develop petals and new internal flowers instead of stamens and carpels, respectively, resulting in sterility (Yanofsky et al. 1990). Limitations in these technologies include non-target effects, variability and incompleteness of knock-downs, and instability over time. These limitations must be addressed, especially if the technologies are to be used in long-lived organisms (Mansoor et al. 2006).

Recently, several precise genome editing tools have been developed using customizable, sequence-specific nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (Cas9) (Gaj et al. 2013). Among these genome editing approaches, CRISPR/Cas9 rapidly became the best choice for gene editing in various plant species (reviewed by Schaeffer and Nakata 2015), because of its simplicity, efficiency, and design flexibility. The CRISPR/Cas9 system utilizes a short guide RNA (sgRNA) of about 20 nucleotides that recognizes the target DNA sequence simply via Watson-Crick base pairing. The resulting complex recruits the Cas9 nuclease which induces double-stranded breaks, and, often indel mutations, through an error-prone repair mechanism called non-homologous end joining (NHEJ) (Jinek et al. 2012). To date, a few studies have been reported on the application of CRISPR/Cas9 system to woody plants. As a proof-of-concept, the CRISPR/Cas9 system was used to induce mutations within the target gene *PHYTOENE DESATURASE* (*PDS*) of *Citrus sinensis* that resulted in an albino phenotype (Jia and Wang 2014). Similar result was obtained in *Malus prunifolia* × *M. pumila* (Nishitani et al. 2016), and *Populus tomentosa* (Fan et al. 2015). Zhou et al. (2015) reported a high mutation efficiency targeting two 4-coumarate:CoA ligase (*4CL*) genes, *4CL1* and *4CL2* in *Populus tremula* x *P. alba*, resulting in reduced lignin and tannin content. More recently, CRISPR/Cas9 was utilized for basic research elucidating gene function in *Populus* (Wang et al. 2017; Yang et al. 2017) and for improving disease-resistance in *Citrus paradisi* by inducing mutations in a canker-susceptibility-gene (Jia et al. 2017).

Here I describe the first application of a CRISPR/Cas9 system in black ash (*Fraxinus nigra*). I targeted an *AG* orthologous gene, *FnAG* (Lee and Pijut 2017a). Transgenic black ash lines were produced using a total of five different *Cas9* expression vectors, and mutations from two independent lines that showed one nucleotide substitution were identified. *Cas9* transgene silencing in some transgenic lines did not have any detected mutation events. The low mutation rate (approximately 0.03%) which was, in part, caused by the transgene silencing suggests that further studies should be conducted to find an efficient promoter for stable expression of transgenes in black ash.

5.3 Materials and Methods

5.3.1 Plant material

Mature black ash seeds obtained from the National Tree Seed Centre (Natural Resources Canada, Fredericton, New Brunswick, Canada) were surface disinfested and stratified as described by Beasley and Pijut (2013). Embryo extraction, germination, and hypocotyl-explant preparation followed the procedure described in chapter three. Hypocotyls were used to determine explant sensitivity to hygromycin and for transformation experiments. Briefly, hypocotyls were placed on a modified Murashige and Skoog (1962) (MS; M499, *Phyto*Technology Laboratories, Shawnee Mission, KS) pre-culture medium with organics (100 mg L^{-1} myoinositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCL, 0.1 mg L^{-1} thiamine HCL, and 2 mg L^{-1} glycine), and supplemented with 13.3 µM 6-benzylaminopurine (BA), 4.5 µM thidiazuron (TDZ), 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water (C195, *Phyto*Technology Laboratories) in Petri plates (100 × 25 mm; 45 mL medium). Unless noted otherwise, all media contained 3% (w/v) sucrose and 0.7% (w/v) Bacto agar (No. 214030; Becton Dickinson and Co., Sparks, MD) with the pH adjusted to 5.7 before autoclaving for 20 min at 121°C. All cultures were maintained in a growth room at 24 ± 2°C under a 16 h photoperiod (approximately 80 µmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps.

5.3.2 Effect of hygromycin on hypocotyl explants

The effect of hygromycin on callus induction and shoot formation was investigated to determine the optimum concentration for selection of transformed explants. Seven-day-old hypocotyls were cultured horizontally on MS pre-culture medium with hygromycin (0, 1, 2, 3, 4, 5, 6, or 7 mg L^{-1}) in Petri plates (100 × 25 mm; 45 mL medium). Hygromycin was dissolved in sterile, deionized water and filter-sterilized (0.22 µm) and added to the medium after autoclaving. Hypocotyls were cultured for 3 weeks, transferred to fresh treatment medium, and the regeneration response for callus and shoot induction were recorded after 6 weeks of culture in vitro. Three replicates of 12 hypocotyls each were used for each treatment.

5.3.3 Selection of CRISPR/Cas9 target sites

The exon sequence of *FnAG* (GenBank: KX592173.1; Lee and Pijut 2017a) was entered in the web-based tool CHOPCHOP (Montague et al. 2014) to find the CRISPR/Cas9 target sites.

Three target sequences were selected based on their location within each exon region (Fig. 5.1). Several polymerase chain reaction (PCR) products flanking the target regions were sequenced to find any possible allelic variation or single-nucleotide polymorphisms (SNPs), and the results confirmed that there were no variations within the selected three target sites (data not shown).

5.3.4 Assembly of Cas9/sgRNA expression constructs

5.3.4.1 pCam1380-35S::hSpCas9

The pCam1380-*35S::hSpCas9* vector was constructed following the procedure described by Jia and Wang (2014). The CaMV 35S promoter in pBI121 vector was digested with *Hind*III and *Bam*HI, and ligated into pCAMBIA1380 vector. The human codon-optimized Cas9 gene (*hSpCas9*) was amplified from Addgene (Cambridge, MA, USA) plasmid 42230 (Cong et al. 2013) using two flanking primers with added *Bam*HI and *Eco*RI sites, Cas9-5-BamHI and Cas9-3-EcoRI (all primers are listed in Table 5.1). Unless noted otherwise, all PCR for cloning were conducted using proof-reading DNA polymerase, Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions for PCR mixture preparations, and all cycling programs consisted of an initial denaturation at 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 1-3 min, and a final extension at 72°C for 10 min. The PCR product was digested with *Bam*HI and *Eco*RI, and inserted downstream of the CaMV 35S promoter in pCAMBIA1380. The amplicon of Nos terminator, obtained from pBI121 vector by PCR using the primers NosT-5-EcoRI and NosT-3-XhoI-AscI, was inserted downstream of the Cas9 coding region following digestion with *Eco*RI and *Asc*I to create p1380-Cas9. The sgRNA scaffold sequence was amplified from Addgene plasmid 41819 (Mali et al. 2013) using two flanking primers with added *Bam*HI and *Sac*I sites, sgRNA-5-BamHI and sgRNA-3-SacI. The amplicon was digested with *Bam*HI and *Sac*I, and inserted into pBI121 replacing GUS reporter gene. PCR was used to amplify the 35S-sgRNA fragment using the primers 35S-5-XhoI and sgRNA-AG#-R, and the sgRNA-NosT fragment using the primers NosT-3-AscI and sgRNA-AG#-F. Primers sgRNA-AG1 and sgRNA-AG2 were for target 1 and 2, respectively (Fig. 5.1). To clone the sgRNA portion into p1380-Cas9 vector using three-way ligation, *Xho*I-digested 35S-sgRNA fragment, *Asc*I-treated sgRNA-NosT fragment, and *Xho*I*Asc*I-double-digested 1380-Cas9 were mixed together in a single tube and treated with T4 DNA ligase (NEB). Two final vectors of pCam1380-*35S::hSpCas9* were constructed targeting *FnAG* gene (Fig. 5.2a).

5.3.4.2 pCam1300-pAtUBQ::hSpCas9

The pUC-18 vector harboring sgRNA scaffold, driven by *Arabidopsis U6* promoter (*pAtU6*), and *hSpCas9* under the control of *Arabidopsis UBIQUITIN* promoter (*pAtUBQ*) was developed and kindly provided by Dr. Zhu (Purdue University). To clone target sequences between the *pAtU6* and sgRNA scaffold region, each pair of the phosphorylated oligonucleotides (AG#-1-Phos and AG#-2-Phos) were annealed and ligated into *Bbs*I-treated pUC-18. Primers AG2-1/2-Phos and AG3-1/2-Phos were for target 2 and 3, respectively (Fig. 5.1). The construct was then digested with *Kpn*I and *Hind*III-HF, separated on 1% (w/v) agarose gel stained with 0.3 μg mL^{-1} ethidium bromide, and 5.7-kb bands were recovered to obtain sgRNA-Cas9 fragment. The purified 5′-*Kpn*I-sgRNA-Cas9-*Hind*III-3′ fragment was inserted into *Kpn*I*Hind*III-double-digested pCAMBIA1300 to create two final vectors of pCam1300-*pAtUBQ::hSpCas9* targeting *FnAG* gene (Fig. 5.2b).

5.3.4.3 pKSE401-35S::zCas9

The pKSE401-*35S::zCas9* was constructed as described by Xing et al. (2014). PCR was carried out using pCBC-DT1DT2 vector (Addgene plasmid 50590) with primers AG-DT1-BsF, AG-DT1-F0, AG-DT2-R0, and AG-DT2-BsR, to amplify *pAtU6-26::sgRNA1:pAtU6-29:sgRNA2* fragment. The purified PCR product was then used for Golden Gate reaction with the pKSE401 vector (Addgene plasmid 62202) that harbored *Zea mays* codon-optimized *Cas9* driven by CaMV 35S promoter, T4 DNA ligase (high concentration, NEB), and *Bsa*I. The reaction condition consisted of an incubation at 37°C for 5 h, followed by 5 min at 50°C, and finalized at 80°C for 10 min. The final pKSE401-*35S::zCas9* vector contained two sgRNAs in tandem targeting two sites (targets 1 and 2) simultaneously (Fig. 5.2c).

5.3.5 *Agrobacterium*-mediated transformation and regeneration of putative transformants

The transformation and regeneration of putative transformants followed the procedures described in chapter three. Briefly, the procedures consisted of: (1) Inoculation of single colonies of *Agrobacterium tumefaciens* strain EHA105 harboring each vector in 20 mL liquid YEP medium (10 g L^{-1} yeast extract, 10 g L^{-1} bactopeptone, and 5 g L^{-1} NaCl, at pH 7) containing appropriate antibiotics (20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin or 50 mg L^{-1} hygromycin) and cultured in the dark for 2 days at 28°C; (2) Sonication-treated hypocotyls were immersed in

Agrobacterium suspension (OD_{600} = 0.9-1.0) and vacuum-infiltrated for 10 min; (3) Co-cultivation on semi-solid MS medium (13.3 μM BA, 4.5 μM TDZ, 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water) for 2-3 days in the dark at 28°C; (4) Selection and regeneration of putative transformants on MS selection medium (13.3 μM BA, 4.5 μM TDZ, 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water, 300 mg L^{-1} timentin, and 40 mg L^{-1} kanamycin or 5 mg L^{-1} hygromycin). The kanamycin- or hygromycin-resistant explants with adventitious shoot buds continued to grow on selection medium without TDZ in order to prevent shoot elongation inhibition. Once the insertion of *Cas9* expression cassette was confirmed by PCR, the transgenic shoots were regularly subcultured to fresh medium without kanamycin or hygromycin every 3 weeks. Elongated microshoots (3-4 cm in length) were induced to form roots on woody plant medium (WPM; Lloyd and McCown 1980) supplemented with 5.7 μM indole-3-acetic acid and 4.9 μM indole-3-butyric acid in Magenta™ GA-7 vessels as previously described (Beasley and Pijut 2013; Lee and Pijut 2017b). Rooted plantlet was then acclimatized in the greenhouse.

5.3.6 Amplicon-sequencing

Genomic DNA was extracted from the leaves of non-transformed control shoots and the regenerated, putative transformants following the procedure described by Lefort and Douglas (1999). A 621-bp fragment of *hSpCas9* and a 280-bp fragment of *zCas9* were amplified to confirm the presence of *Cas9* expression cassette in putative transformants. The PCR reaction mix was prepared in a total volume of 25 μL containing 1 μL genomic DNA (100-200 ng), 12.5 μL One *Taq* Hot Start 2× Master Mix (NEB), 1 μL each of 10 μM hCas9-F and hCas9-R or 10 μM zCas9-F and zCas9-R primers (Table 5.1), and sterile-deionized water was added to make the final volume. All cycling programs consisted of an initial denaturation at 94°C for 30 s, followed by 35 cycles of 94°C for 15 s, 55°C for 15 s, 68°C for 30 s, and a final extension at 68°C for 5 min. Amplicons were visualized under UV light after gel electrophoresis (1% w/v agarose plus 0.3 μg mL^{-1} ethidium bromide).

Genomic DNA extracted from PCR-positive transgenic lines was used to confirm the integration of the *Cas9* expression cassette and then used as a template to amplify a fragment of the endogenous target gene (*FnAG*) using primers flanking the target sites: FnAG-1 and FnAG-2 for targets 1 and 2; and FnAG-3 and FnAG-4 for target 3 (Table 5.1). The proof-reading DNA polymerase, Phusion High-Fidelity DNA polymerase (NEB) was used as described previously,

and the cycling programs consisted of an initial denaturation at 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Amplicons were separated on 2% (w/v) agarose gel stained with 0.3 μg mL^{-1} ethidium bromide, and bands were recovered and sequenced. All sequencing results were compared with the reference sequence of *FnAG* to identify mutations.

5.3.7 Expression analysis

Total RNA was extracted from leaves of non-transformed control shoots and the transgenic lines using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. DNase I (Thermo Fisher Scientific, Grand Island, NY, USA) -treated total RNA (1 μg) was used to synthesize the first-strand cDNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and an oligo-dT primer. To analyze the transcript expression level of *Cas9* transgene, semi-quantitative reverse transcription (RT)-PCR was conducted. Black ash *PDS* (*FnPDS*) was used as a reference gene. *FnPDS* was identified in a black ash genome sequence database (http://www.ashgenome.org) by BLAST search using *P. trichocarpa PDS* (Potri.014G148700) sequence. The PCR reaction mix was prepared in a total volume of 25 μL containing 1 μL cDNA, 12.5 μL One *Taq* Hot Start 2× Master Mix (NEB), 1 μL each of 10 μM hCas9-F and hCas9-R or 10 μM zCas9-F and zCas9-R or 10 μM FnPDS-F and FnPDS-R primers (Table 5.1), and sterile-deionized water was added to make the final volume. The PCR cycling conditions were as follows: 94°C for 30 s, followed by 35 cycles of 94°C for 15 s, 55°C for 15 s, 68°C for 30 s, and a final extension at 68°C for 5 min. The PCR products were visualized under UV light after gel electrophoresis (1% w/v agarose plus 0.3 μg mL^{-1} ethidium bromide).

5.4 Results and Discussion

5.4.1 Effect of hygromycin on hypocotyl explants

Black ash hypocotyls were highly sensitive to hygromycin. Callus formation was significantly reduced at 5 mg L^{-1} hygromycin and completely inhibited at ≥ 6 mg L^{-1} (Table 5.2). Adventitious shoot induction was significantly affected at 3 mg L^{-1} hygromycin and no shoots were regenerated when hypocotyls were exposed to ≥ 4 mg L^{-1} hygromycin (Table 5.2). The results indicated that black ash hypocotyls were more sensitive to hygromycin in comparison to

kanamycin; 40 mg L^{-1} kanamycin was used to select transformed explants in our earlier study (chapter three). We determined 5 mg L^{-1} hygromycin to be the optimal concentration for screening transformed hypocotyl explants.

5.4.2 sgRNA design and CRISPR/Cas9 vector construction

An *AG* ortholog in black ash, *FnAG*, was selected as the target of genome editing. Three independent sgRNAs with 19-20 bp in length were designed based on their location within the exon regions (Fig. 5.1). Potential target sequences in the first exon were excluded in case proteins without the first exon are partially functional. Target 1 (5′-TGCTCAGATTCCTCAAACAA-3′) was located in the 3rd exon, a region responsible for an intervening domain that functions for the selective formation of a DNA-binding dimer. Target 2 (5′-GAGGTTCTTGAGTTCTCTG-3′) was in the 5th exon in a location translated into a keratin domain involved in protein-protein interaction. Target 3 (5′-GAAAACACTACAAATCGTC-3′) was located in the 2nd exon where it was responsible for the formation of MADS domain which determines DNA-binding.

Three different backbone of vectors were used in this study to express Cas9 and sgRNAs for genome editing. Two pCam1380-*35S::hSpCas9* vectors were constructed to generate sgRNAs for targets 1 and 2, respectively; two pCam1300-*pAtUBQ::hSpCas9* vectors were cloned to express sgRNAs for targets 2 and 3, respectively; and one pKSE401-*35S::zCas9* vector was assembled to produce sgRNA for targets 1 and 2, simultaneously (Fig. 5.2). Therefore, a total of five binary vectors were constructed to generate mutations in *FnAG*.

In order to produce an optimal hairpin structure of sgRNA, which combines the essential components of the crRNA and tracrRNA into a single molecule (Josephs et al. 2015), the U6 promoter is commonly used. The RNA polymerase III-dependent U6 promoter generates small nuclear RNAs such as short hairpin RNA without forming 5′-cap or 3′-poly(A) tail (Xia et al. 2003), and this feature makes U6 promoter ideal for expressing sgRNAs in CRISPR/Cas9 system. The only requirement for use of the U6 promoter was that the first nucleotide in the sgRNA should be a guanine (G) (Fichtner et al. 2014). However, this restriction can be bypassed by simply adding the extra G to the 5′ end of the target sequence. In the present study, an additional G was added to the 5′ end of the target 1 sequence which started with thymine (T), in order to ensure its expression under the *pAtU6* promoter in pKSE401-*35S::zCas9* vector. Otherwise, the CaMV 35S promoter was used to express sgRNA in pCam1380-*35S::hSpCas9* vector. Jia and Wang (2014) reported a

successful application of CRISPR/Cas9 system to sweet orange via transient co-expression of sgRNA and Cas9 driven by CaMV 35S promoter, indicating the CaMV 35S promoter also works to produce functional sgRNA.

5.4.3 Regeneration of transgenic lines and confirmation of mutations

Putatively transformed explants were selected on MS medium containing the proper antibiotic (Fig. 5.3a). Once adventitious shoots were induced from the putatively transformed hypocotyls (Fig. 5.3 b, c), genomic DNA was extracted and PCR was used to confirm the presence of a *Cas9* expression cassette. A total of 50 transgenic lines were confirmed by PCR (Fig. 5.3f; Table 5.3). Only one transgenic line (1300T21) successfully produced in vitro roots (Fig. 5.3d), and the rooted plantlet was acclimatized to the greenhouse (Fig. 5.3e). In order to detect mutations in the 50 transgenic lines, PCR amplicons were obtained using primers flanking the target sites, and the amplicons were sequenced. The results showed that only two transgenic lines produced using the pCam1300-*pAtUBQ::hSpCas9* vector, namely 1300T2-3 and 1300T3-1, were found to have mutant alleles in the *FnAG* target 3 region (Fig. 5.4; Table 5.3). Both mutations were chimeric, with a one nucleotide substitution (thymine to cytosine) two base pairs upstream from the protospacer adjacent motif sequence of only one allele; the other allele had a wild-type sequence. The one nucleotide substitution (T to C) was a silent mutation that caused no amino-acid change, so the mutation was unlikely to produce sterile flowers.

The efficacy of each *Cas9* expression cassette used in this study was previously validated in other plant species including sweet orange, tomato, and *Arabidopsis* (Jia and Wang 2014; Pan et al. 2016; Xing et al. 2014). The pCam1380-*35S::hSpCas9* vector induced various indel mutations on the target gene in sweet orange with a mutation rate of up to 3.9% (Jia and Wang 2014). The pCam1300-*pAtUBQ::hSpCas9* vector, which also induced mutagenesis in black ash, showed a high mutation frequency in the first generation of transgenic tomato plants, with an average frequency of 83.56% (Pan et al. 2016). The pKSE401-*35S::zCas9* construct was applied to *Arabidopsis*, resulting in many different mutated alleles in a single transgenic plant with a high mutation frequency (> 90%) (Xing et al. 2014).

Given the observed low mutagenesis rate in the present study, we could speculate the possibility of low activity of Cas9 proteins and/or low expression of *Cas9* genes. To test if the

expression of *Cas9* genes was low, total RNA was isolated from the transgenic lines that did not contain mutations. The semi-quantitative RT-PCR results revealed that the *Cas9* expression was silenced, while the reference gene *FnPDS* was expressed (Fig. 5.5). Similar transgene silencing was observed in our previous study of transgenic black ash using *Bt-cry8D2*. Zhang et al. (2014) also found that seven of the 57 T_0 transgenic rice plants had no mutation at the target sites even in the presence of the *Cas9* expression cassette, possibly because of the silencing of the *Cas9* and/or sgRNA transgenes. In GM trees, transgene expression was commonly unstable during in vitro culture compared to early field screening (Harfouche et al. 2011). For example, an herbicide-resistant poplar showed stable resistance against glyphosate under field conditions for over 8 years, whereas nearly all were unstable during in vitro culture (Li et al. 2008). As discussed earlier in chapter three, highly sequence-specific DNA methylation of a transgene can be induced by a RNA-directed DNA methylation process, resulting in transgene silencing. Weinhold et al. (2013) reported a successive increase of *de novo* methylation in a CaMV 35S promoter during vegetative growth in *Nicotiana attenuata*, up to 77% absolute increase within 45 days of growth, resulting in a rapid loss of the resistance marker expression and down-regulation of the transgene expression by more than 200-fold in subsequent generation.

5.5 Conclusions

In summary, transgenic black ash shoots and one transgenic plant harboring the *Cas9* expression cassette were developed. Among 50 transgenic lines, only two lines had a mutation in the target site. The low mutation rate (average of 0.03%; Table 5.3) might have been caused by the *Cas9* transgene silencing. Further intensive studies are required to find more efficient promoters for stable transgene expression in transgenic black ash.

Table 5.1. Primer sequences.

Primer	Sequence (5' to 3')
Cas9-5-BamHI	AGGTGGATCCGGTTGGACCGGTGCCACCATG
Cas9-3-EcoRI	TGATCAGCGAGCTCTAGGAATTCTTA
NosT-5-EcoRI	AGGATCCACCGGTGCACGAATTCCGAATTTCCCCGATCGTTCAA
NosT-3-XhoI-AscI	AGGCGCGCCATTTAAATCTCGAGCCGATCTAGTAACATAGATGA
sgRNA-5-BamHI	TCGACTGGATCCGGTACCAAG
sgRNA-3-SacI	AGGTGAGCTCTTCGCCCTTTAATGCCAACTT
35S-5-XhoI	ACTCGAGAGATTAGCCTTTTCAAATTTCAGAAAGA
NosT-3-AscI	ACCTGGGCCCGGCGCGCCGATCTAGTAACATAGATGA
sgRNA-AG1-R	AATCTGAGCACGTGTTCTCTCCAAATGAAAT
sgRNA-AG1-F	CCTCAAACAAGTTTTAGAGCTAGAAATAGCAA
sgRNA-AG2-R	AAGAACCTCGCGTGTTCTCTCCAAATGAAAT
sgRNA-AG2-F	GAGTTCTCTGGTTTTAGAGCTAGAAATAGCAA
AG2-1-Phos	GATTGAGGTTCTTGAGTTCTCTG
AG2-2-Phos	AAACCAGAGAACTCAAGAACCTC
AG3-1-Phos	GATTGAAAACACTACAAATCGTC
AG3-2-Phos	AAACGACGATTTGTAGTGTTTTC
AG-DT1-BsF	ATATATGGTCTCGATTGGCTCAGATTCCTCAAACAAGTT
AG-DT1-F0	TGGCTCAGATTCCTCAAACAAGTTTTAGAGCTAGAAATAGC
AG-DT2-R0	AACCAGAGAACTCAAGAACCTCCAATCTCTTAGTCGACTCTAC
AG-DT2-BsR	ATTATTGGTCTCGAAACCAGAGAACTCAAGAACCTCCAA
hCas9-F	TTACCCTGGCCAACGGCGAG
hCas9-R	GCTGTTTCTGCTCATTATCC
zCas9-F	AAGCTGTTCATCCAGCTCGT
zCas9-R	CCAGGTCATCGTCGTATGTG
FnAG-1	CAAATTGTTGATGTCCAATGGT
FnAG-2	TGCATGTACTCAATTTCAGCAA
FnAG-3	ATGGCATTGCAGAGTGATCA
FnAG-4	AACAAAAGTGCCCTAAGAACAAAG
FnPDS-F	ATTCTCCACGTCCAACCAAA
FnPDS-R	CTGGCTTGTTTGGCATTGCA

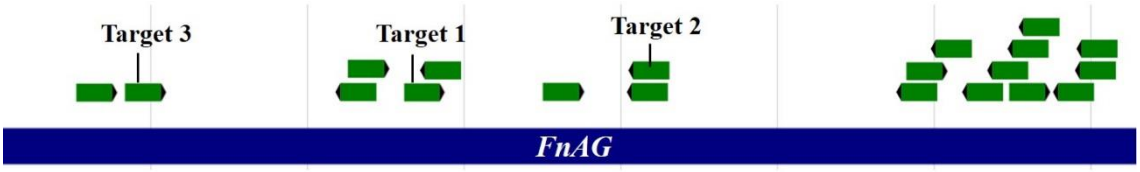
Table 5.2. Effect of hygromycin concentration on percent callus formation and adventitious shoot regeneration of black ash hypocotyls.

Hygromycin ($mg\ L^{-1}$)	Callus Formation[a] (%)	Shoot Induction[a] (%)
0	57.8 ± 5.9a	40.0 ± 3.8a
1	46.7 ± 6.7a	24.4 ± 5.9b
2	48.9 ± 2.2a	13.3 ± 3.8c
3	17.8 ± 4.4b	4.4 ± 2.2d
4	6.7 ± 3.8bc	0d
5	2.2 ± 2.2c	0d
6	0c	0d
7	0c	0d

[a] Values represent means ± SE; followed by the same letter in the same column were not significantly different by the Duncan's multiple comparison test ($p < 0.05$).

Table 5.3. Summary of transformation and mutation events using each *Cas9* expression vectors.

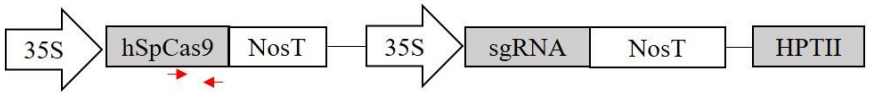
Vector	Target	No. of explants	No. of transgenic lines	Transformation frequency (%)	Mutation rate (%)
pCam1380-*35S::hSpCas9*	*FnAG-1*	522	7	1.34%	0.00%
	FnAG-2	416	3	0.72%	0.00%
pCam1300-*pAtUBQ::hSpCas9*	*FnAG-2*	2098	16	0.76%	0.00%
	FnAG-3	2135	13	0.61%	15.38%
pKSE401-*35S::zCas9*	*FnAG-2&3*	898	11	1.22%	0.00%
Total		6069	50	0.82%	0.03%



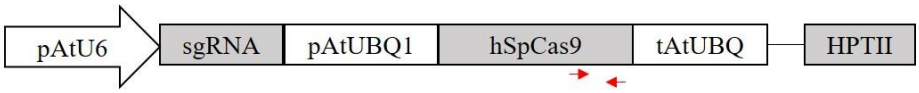
Target 1: 5′-TGCTCAGATTCCTCAAACAATGG-3′
Target 2: 5′-GAGGTTCTTGAGTTCTCTGAGG-3′
Target 3: 5′-GAAAACACTACAAATCGTCAGG-3′

Figure 5.1. Schematic diagram of *FnAG* exon regions. Arrows show all possible target sites containing an NGG protospacer adjacent motif. Target sites were searched using a web-based tool CHOPCHOP (Montague et al. 2014).

(a) pCam1380-*35S::hSpCas9*



(b) pCam1300-*pAtUBQ::hSpCas9*



(c) pKSE401-*35S::zCas9*

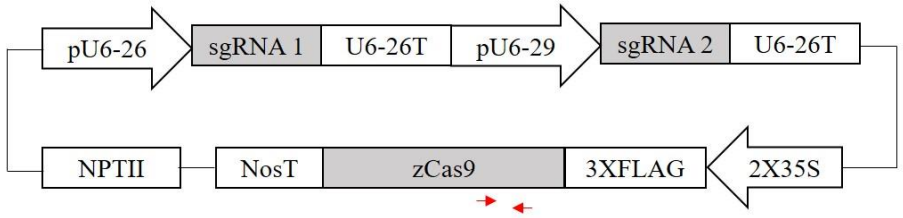


Figure 5.2. Schematic diagram of Cas9 and sgRNA expression cassettes. (a) pCAMBIA1380 vector harboring *hSpCas9* (humanized *Streptococcus pyogenes Cas9*) and sgRNA scaffold, driven by CaMV35S promoter, and *hptII* for hygromycin selection; (b) pCAMBIA1300 vector harboring *hSpCas9* driven by *Arabidopsis U6* promoter, sgRNA scaffold driven by *Arabidopsis UBIQUITIN* promoter, and *hptII*; and (c) pKSE401 vector harboring *zCas9* (*Zea mays* codon-optimized *Cas9*) driven by CaMV35S promoter, *nptII* for kanamycin selection, and two sgRNA scaffolds driven by *Arabidopsis U6-26* and *U6-29* promoters, respectively. Arrows indicate binding sites of the primers using for PCR amplification.

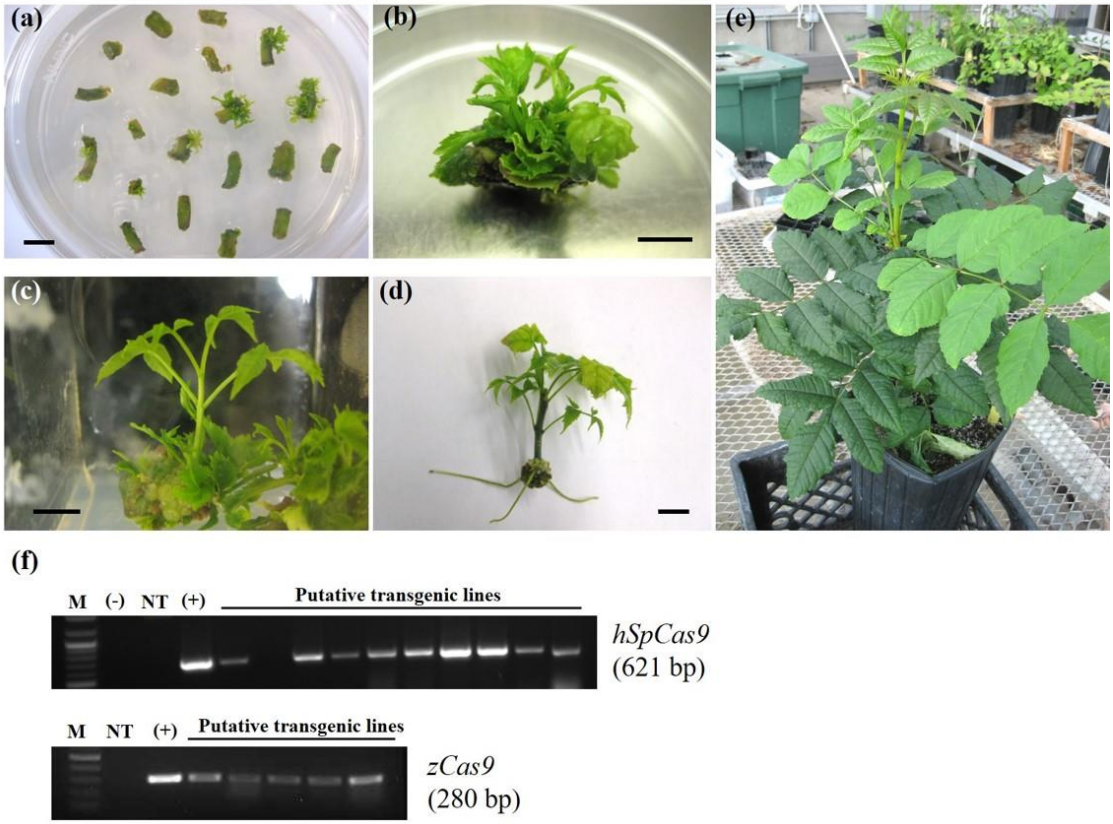


Figure 5.3. Regeneration of transgenic *Fraxinus nigra* (black ash). (a) In vitro selection of putative transformed hypocotyls on medium containing proper selection agents (*bar* = 1 cm); (b) Adventitious shoot bud induction from putative transformed hypocotyls (*bar* = 1 cm); (c) Elongated transgenic shoot (*bar* = 1 cm); (d) In vitro root production (*bar* = 1 cm); (e) Acclimatized transgenic black ash plant in the greenhouse; and (f) Detection of a 621-bp fragment of *hSpCas9* and a 280-bp fragment of *zCas9* via PCR from the leaves of putative transgenic lines. M, 100-bp DNA ladder; (-), water control; NT, negative control of non-transformed black ash DNA; (+), positive vector control.

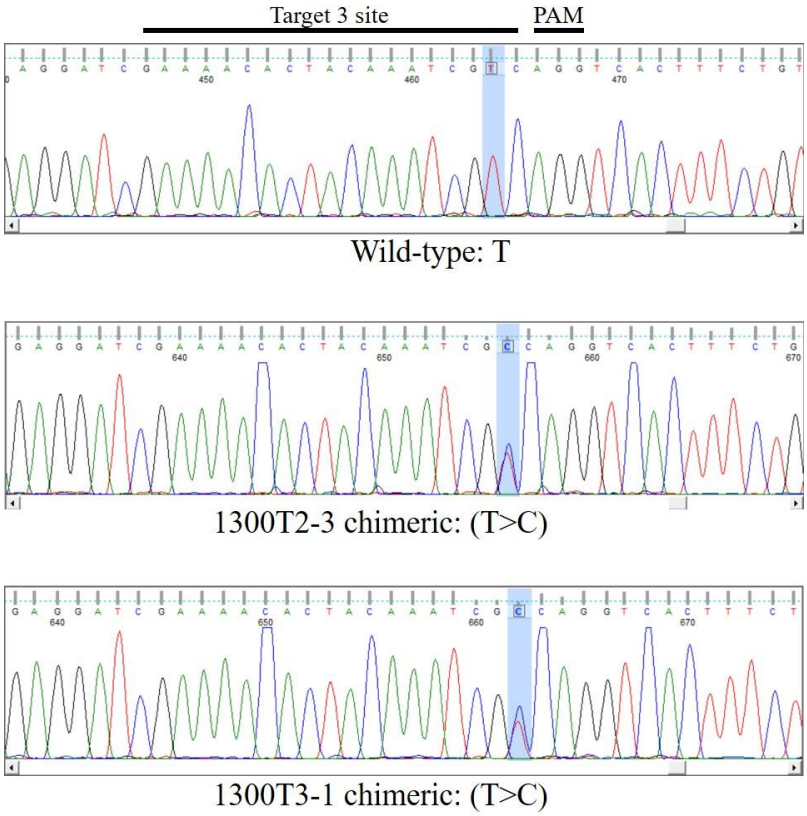


Figure 5.4. Illustration of the results of sequencing target sites. Wild-type sequence showed a thymine at two base pairs upstream from protospacer adjacent motif, whereas the two transgenic lines (1300T2-3 and 1300T3-1) showed both thymine and cytosine, indicating a chimeric mutation

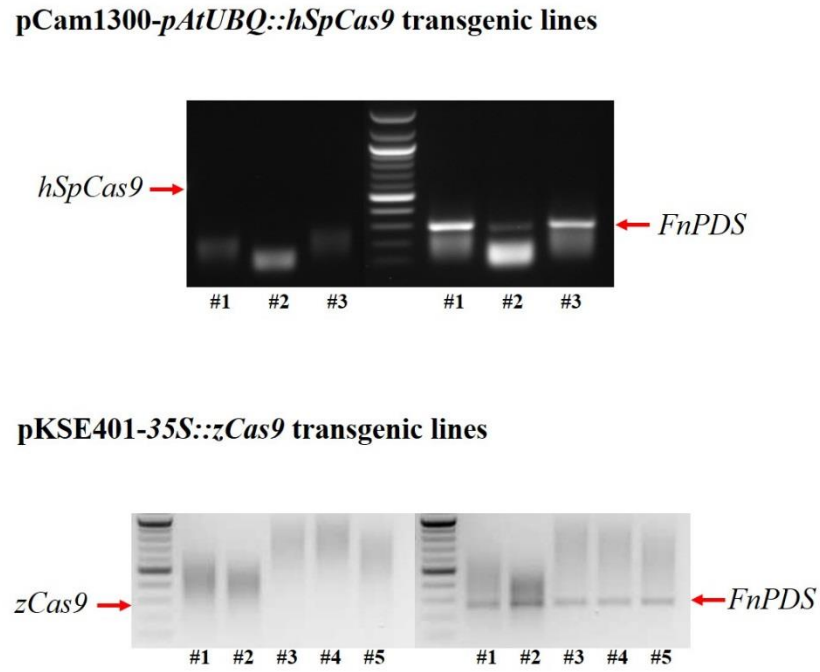


Figure 5.5. Semi-quantitative RT-PCR analysis for detecting *Cas9* transcript expression in the leaves of transgenic lines. The expression of *hSpCas9* (a) and *zCas9* (b) transcripts were not detected, but expression of the reference gene *PHYTOENE DESATURASE* (*FnPDS*) was observed

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Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N, others (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12:797–807.

Zhou X, Jacobs TB, Xue L-J, Harding SA, Tsai C-J (2015) Exploiting SNPs for biallelic CRISPR mutations in the outcrossing woody perennial *Populus* reveals 4-coumarate:CoA ligase specificity and redundancy. New Phytol 208:298–301.

VITA

Junhyung Lee

Research Interests
Genome editing, Molecular biology, Biotechnology, Genetics, Plant physiology

Education

2017 **Ph.D., Forest Genetics**
Purdue University, Dept. of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center, West Lafayette, IN
Dissertation: *Development of transgenic black ash (Fraxinus nigra) for resistance to the emerald ash borer and genome editing for reproductive sterility*.

2011 **M.S., Horticultural Science**
Seoul National University, Dept. of Plant Science, Seoul, South Korea
Thesis: *Changes of cold hardiness and carbohydrate content in highbush blueberry (Vaccinium corymbosum) during cold acclimation and deacclimation.*

2009 **B.A., Agriculture and Life Science, Cum laude**
Seoul National University, Dept. of Plant Science, Seoul, South Korea
Undergraduate Research Project: *Development of intron-based PCR markers (SCAR) and construction of inter-specific linkage map in Capsicum*.

Professional Experience
2013-present Graduate Research Assistant (Ph.D.), Purdue University, Dept. of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center.

Genome editing
- Applied CRISPR/Cas9 system to induce knockout mutations in a flowering control gene, which may result in reproductive sterility, minimizing the potential for transgene flow.
- Constructed a series of sgRNA/Cas9 cassettes for multiplex editing.

Genetic transformation
- Optimized *Agrobacterium*-mediated transformation system for a forest tree species Fraxinus nigra.
- Developed a protocol to produce insect-resistant black ash by introducing *Bt-Cry8D2* gene and analyzed the transgene expression in transcript and protein levels by qPCR and Western, respectively.
- Estimated the transgene copy number by qPCR.

Gene cloning
- Isolated a floral homeotic gene in Fraxinus nigra by RT-PCR and RACE.
- Characterized the gene function by ectopic expression in transgenic *Arabidopsis*.

Plant tissue culture

- Developed a plant regeneration protocol using leaf-explants.
- Routinely performed plant tissue culture to induce adventitious shoot/root.

2012-2013 Graduate Research Assistant (Ph.D.), Purdue University, Dept. of Horticulture and Landscape Architecture.

Protein-DNA/protein-protein interaction

- Performed EMSA to test the DNA binding specificity of the transcription factor GTL1 using single amino-acid substitution mutations.
- Conducted pull-down assay to find proteins that interact with GTL1.

2011-2012 Research Scientist, Seoul National University, Seoul, South Korea
Research Institute for Agriculture and Life Sciences.

Plant physiology study

- Evaluated cold hardiness in 21 highbush blueberry cultivars using electrolyte leakage analysis.
- Demonstrated the effect of soluble sugar content on the level of cold hardiness.
- Analyzed the gene expression of beta-amylase that played a role in the conversion from starch to soluble sugar during cold acclimation.

Teaching Experience

2015 Fall Teaching Assistant, Purdue University, Dept. of Forestry and Natural Resources, FNR 331, Forest Ecosystems.

Publications

Lee, J.H. and Pijut, P.M. (In Preparation). Targeted genome editing for gene containment in transgenic black ash (*Fraxinus nigra*).

Lee, J.H. and Pijut, P.M. (In Preparation). Optimization of *Agrobacterium-mediated* genetic transformation of black ash (*Fraxinus nigra*) and development of black ash for emerald ash borer-resistance.

Lee, J.H. and Pijut, P.M. 2017. Adventitious shoot regeneration from in vitro leaf explants of *Fraxinus nigra*. ***Plant Cell Tissue and Organ Culture-Journal of Plant Biotechnology*** 130:335-343.

Lee, J.H. and Pijut, P.M. 2017. Isolation and characterization of a floral homeotic gene in *Fraxinus nigra* causing earlier flowering and homeotic alterations in transgenic *Arabidopsis*. ***Plant Gene*** 10:17-25.

Lee, J.I., Yu, D.J., **Lee, J.H.**, Kim, S.J., and Lee, H.J. 2013. Comparison of mid-winter cold-hardiness and soluble sugars contents in the shoots of 21 highbush blueberry (*Vaccinium corymbosum*) cultivars. ***The Journal of Horticultural Science and Biotechnology*** 88:727-734.

Lee, J.H., Yu, D.J., Kim, S.J., Choi, D., and Lee, H.J. 2012. Intraspecies differences in cold hardiness, carbohydrate content and β-amylase gene expression of *Vaccinium corymbosum* during cold acclimation and deacclimation. ***Tree Physiology*** 32:1533-1540.

Professional Papers, Presentations, and Posters

Lee, J.H. and Pijut, P.M. 2017. Efficient *Agrobacterium*-mediated transformation of black ash (*Fraxinus nigra*) for EAB resistance. *Purdue University Annual Research Symposium*, Dept. of Forestry and Natural Resources, West Lafayette, IN.

Lee, J.H. and Pijut, P.M. 2016. Can we save ash trees from the emerald ash borer? Biotechnology may be the answer. *University and Industry Consortium at Dow AgroSciences*, Indianapolis, IN.

Lee, J.H. and Pijut, P.M. 2016. In vitro plant regeneration from leaf explants of black ash. *Emerald Ash Borer Research and Technology Development Meeting*, Wooster, OH. (Abstract In Press).

Lee, J.H. and Pijut, P.M. 2016. CRISPR/Cas9: Targeting a floral homeotic gene in black ash. *Purdue University Annual Research Symposium*, Dept. of Forestry and Natural Resources, West Lafayette, IN. 2nd place in the PhD Research Competition.

Lee, J.H. and Pijut, P.M. 2015. Isolation and functional characterization of an *AGAMOUS* homolog from black ash (*Fraxinus nigra*). *Society for In Vitro Biology*, Tucson, AZ. *In Vitro Cellular and Developmental Biology-Plant* P-2015, S54.

Lee, J.H. and Pijut, P.M. 2015. Can we save ash trees from the emerald ash borer? – Biotechnology may be the answer. *Indiana Academy of Science*, 130th Annual Meeting, Indianapolis, IN.

Lee, J.H. and Pijut, P.M. 2015. Molecular approaches for development of EAB-resistant black ash. *Emerald Ash Borer National Research and Technology Development Meeting*, Wooster, OH. USDA Forest Service, APHIS, and The Ohio State University, Buck, J., Parra, G., Lance, D., Reardon, R., and Binion, D. (compilers). p. 68, FHTET-2015-07.

Lee, J.H. and Pijut, P.M. 2014. Targeted genome editing for gene containment in transgenic black ash. *Purdue University Annual Research Symposium*, Dept. of Forestry and Natural Resources, West Lafayette, IN.

Lee, J.H. and Pijut, P.M. 2014. Genetic transformation for resistance to the emerald ash borer and isolation of an AGAMOUS homolog in black ash. *IUFRO - Restoring Forests: What constitutes success in the 21st century?* Lafayette, IN.

Lee, J.H., C.Y. Yoo, M.V. Mickelbart, and Hasegawa, P.M. 2013. Proteomic analysis of GTL1 interacting proteins involved in regulating of water use efficiency through the repression of *STOMATAL DENSITY AND DISTRIBUTION 1*. *Dept. of Horticulture and Landscape Architecture Research Retreat*, Purdue University, West Lafayette, IN.

Lee, J.H., Yoo, C.Y., Mickelbart, M.V., and Hasegawa, P.M. 2012. E116 in the GTL1 transcription factor interacts with the GGTAAA-box in *SDD1* promoter and increases stomatal density and plant water use. *Plant Science Social Network*, Purdue University, West Lafayette, IN.

Lee, J.H., Yu, D.Y., Kim, S.J., and Lee, H.J. 2011. Estimation of cold hardiness in floral buds and shoots of highbush blueberries (*Vaccinium corymbosum*). *Conference of the Korean Society for Horticultural Science*, Jeju, South Korea. *Korean Journal of Horticultural Science and Technology*, 29(Sup1):47.

Lee, J.H., Yu, D.Y., Kim, S.J., and Lee, H.J. 2011. Changes of cold hardiness and carbohydrate content during cold acclimation in highbush blueberries (*Vaccinium corymbosum*). *Conference of the Korean Society for Horticultural Science*, Jeju, South Korea. *Korean Journal of Horticultural Science and Technology*. 29(Sup1):47.

Extension Activities

Lee, J.H. and Pijut, P.M. 2015. Integration of molecular biotechnologies for developing insect resistance and flowering control in black ash. *Hardwood Tree Improvement and Regeneration Center Advisory Committee Meeting*, West Lafayette, IN.

Lee, J.H. and Pijut, P.M. 2014. Targeted genome editing for gene containment in transgenic black ash. *Hardwood Tree Improvement and Regeneration Center Advisory Committee Field Day*, West Lafayette, IN.

Lee, J.H. and Pijut, P.M. 2013. Development of black ash (*Fraxinus nigra*) for reproductive sterility and resistance to the emerald ash borer (EAB). *Research presentation given to visiting scientists from Yunnan Academy of Forestry*, Yunnan, China and Tengchong Forestry Bureau, Tengyue Town, Tengchong County, Yunnan, China.

Awards and Fellowships

2013-2017 **PhD Graduate Research Assistantship**, Purdue University, Dept. of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center.

2016 **PhD Research Poster Award, 2nd Place**, Purdue University, Dept. of Forestry and Natural Resources.

2011 **Outstanding Oral Presentation Award**, Conference of the Korean Society for Horticultural Science.

2011 **Lee Chul Young Scholarship**

2007-2011 **Seoul National University, Development Fund Scholarship**

2008 **The Conference of the Korean Agriculture Policy Award**, Competitive Exhibition of Undergraduate Student Research Paper.

2008 **Korea Student Aid Foundation Scholarship**

2004-2005 **Seoul National University, Development Fund Scholarship**