Targeted Genome Editing for Gene Containment in Transgenic Black Ash

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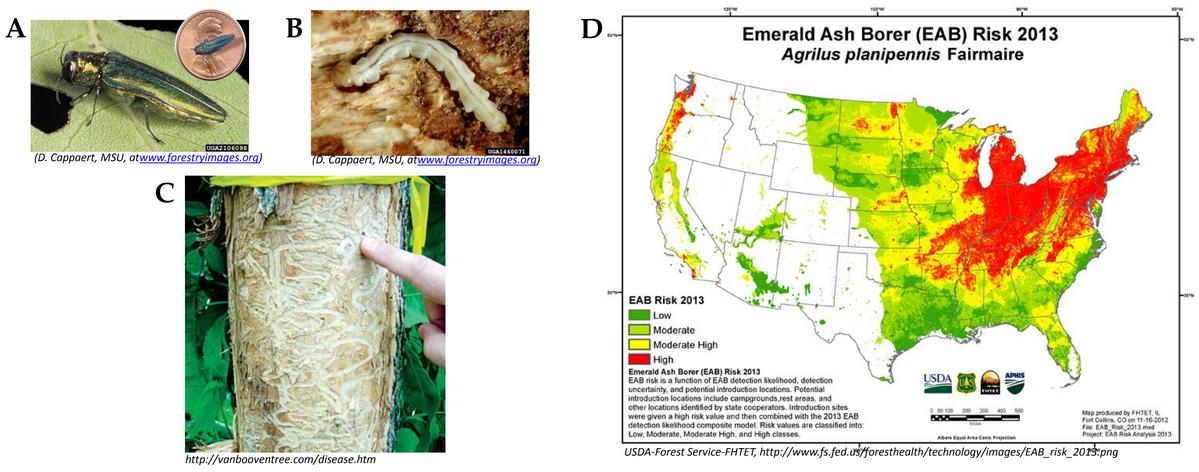
ABSTRACT

Black ash (*Fraxinus nigra*) is valued not only for commercial hardwood applications such as cabinets, paneling, flooring, and veneer, but also for food and habitat for wildlife. The wood is preferred by Native Americans for making splints for basketry. However, the emerald ash borer (EAB), an exotic wood-boring beetle from Asia, has killed millions of ash trees in Michigan since 2002, and EAB has spread to 22 states in the United States, and into Canada. Although several insecticides have been developed to control EAB, it has limitations. As a long-term alternative, development of transgenic black ash with EAB-resistance is urgently needed. A naturally occurring toxin gene from *Bacillus thuringiensis (Bt)* was introduced into the black ash genome through *Agrobacterium*-mediated transformation using hypocotyl explants. Adventitious shoots were regenerated from transformed cells showing kanamycin-resistance, and the presence of the *Bt*-gene was confirmed. Once roots are formed on these shoots, the transgenic plantlets will be acclimatized to the greenhouse. However, transgenic trees are not allowed to be routinely planted because of the potential environmental impacts of transgene flow; movement of genes from a genetically modified organism to its wild or native relatives through pollen. With current molecular technologies, gene containment can be achieved by interfering with flowering. Transcription activator-like effector nuclease (TALEN) is one powerful tool for genome editing by inducing DNA double-strand breaks that stimulate non-homologous end joining or homologous recombination at specific genomic locations. TALENs are artificial restriction enzymes generated by fusing a TALE DNA binding domain of *Xanthomonas* to a DNA cleavage domain of *Fok*I endonuclease. To disrupt black ash *AGAMOUS*, a C-class floral organ identity gene responsible for stamens and carpels, we can manipulate the DNA binding domain of TALEN based on the sequence of black ash *AGAMOUS*. Small insertion or deletion mutations at the target might be induced, so that the *AGAMOUS* gene would be disrupted. As a result, transgenic black ash would be sterile with no stamens and carpels. Our results will help improve our understanding of the usefulness of TALEN technology in the genetic modification of tree species.

OBJECTIVE

To develop transgenic black ash for reproductive sterility and resistance to emerald ash borer

Emerald ash borer (EAB) is threatening North American ash trees



(D. Cappaert, MSU, at www.forestryimages.org)

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http://vanbooventree.com/disease.htm

USDA-Forest Service-FHTET, http://www.fs.fed.us/foresthealth/technology/images/EAB_risk_2013.png

Figure 1. EAB adult **(A)** and larvae **(B). (C)** Larvae bore through the bark into the phloem disrupting the flow of nutrients, resulting in the death of the tree. **(D)** EAB has spread to 22 states in the United States.

Bacillus thuringiensis (*Bt*) toxin gene can be introduced into the black ash genome through *Agrobacterium*-mediated transformation

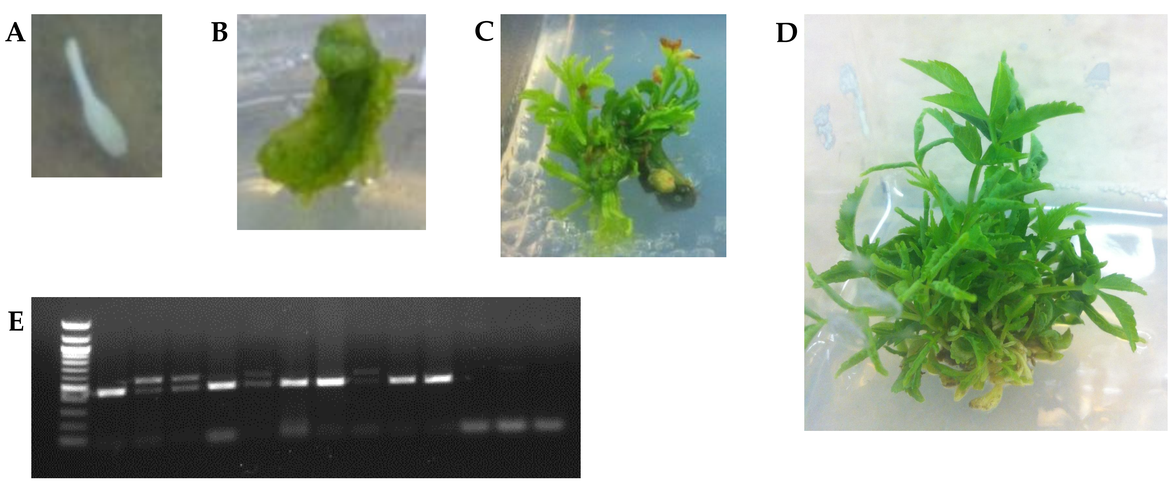
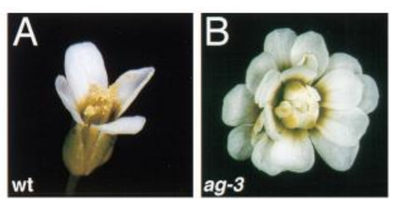


Figure 2. Black ash hypocotyl **(A)** was used for *Agrobacterium*-mediated transformation to introduce the *Bt* gene and selection marker gene. Callus was induced from infected hypocotyl **(B)** and shoots resistant to kanamycin **(C)** were regenerated. Adventitious shoots **(D)** were cultured on shoot elongation medium. **(E)** The presence of the *Bt*-gene in putative transgenic shoots was confirmed by PCR.

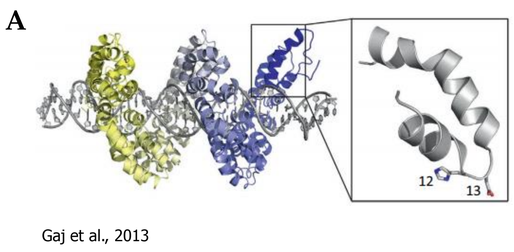
Reproductive sterility can be obtained by disrupting the flowering control gene, *AGAMOUS*



Riechmann et al., 1999

Figure 3. *Arabidopsis* flower of wild-type **(B)** and *agamous* mutant **(B)**. *AGAMOUS* is a C-class floral organ identity gene responsible for stamens and carpels. In flowers of *agamous* mutant plants, stamens and carpels are replaced by petals and a new flower, respectively, resulting in sterility.

Transcription activator-like effector nuclease (TALEN)



Gaj et al., 2013

B

RVD	NI	HD	NG	NH	NN	NS
DNA specificity	A	C	T	G	AG	AC GT

Figure 4. TALENs are artificial restriction enzymes generated by fusing a TALE DNA binding domain to a DNA cleavage domain of FokI endonuclease. TALEs are proteins secreted by *Xanthomonas* that can bind to host plant DNA through a highly conserved repeat domain. **(A)** Residues at 12th and 13th positions of each repeat, called repeat variable diresidue (RVD), determine specific nucleotide on target DNA **(B)**. FokI endonuclease is a non-specific DNA cleavage enzyme and it functions as a dimer.

Transgenic black ash genome can be engineered for reproductive sterility by TALEN

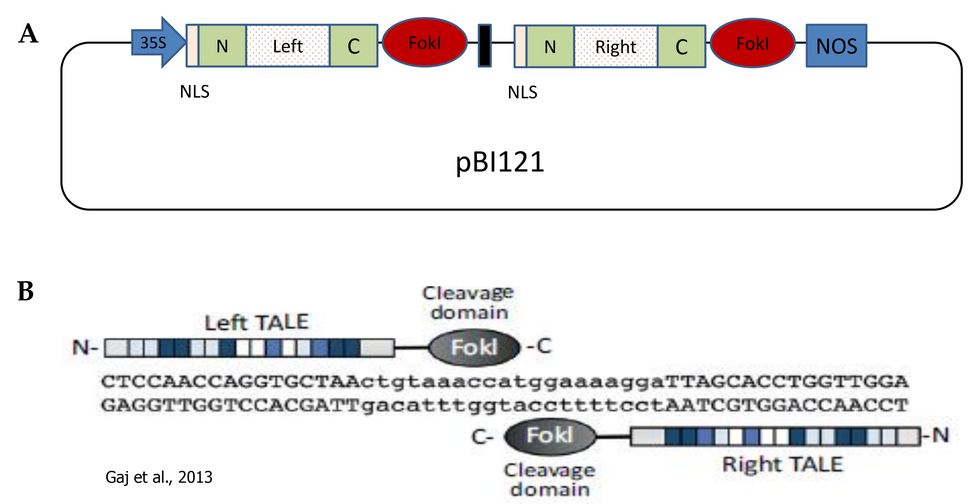


Figure 5. (A) Schematic overview of TALEN construct targeted to black ash *AGAMOUS*. TALEN construct will be introduced into *Bt*-black ash genome through *Agrobacterium*-mediated transformation. **(B)** TALEN proteins will be localized to nucleus and bind to *AGAMOUS* region inducing double-strand break (DSB). By non-homologous end joining DNA repair machinery, small insertion or deletion will be induced on the middle of *AGAMOUS* disrupting the gene.

Further study

- Isolation and characterization of black ash *AGAMOUS*
  - Gene cloning
  - Ectopic expression of black ash *AGAMOUS* in *Arabidopsis*
- Cloning and transformation of TALEN
  - Design TALEN construct based on black ash *AGAMOUS* sequence
  - *Agrobacterium*-mediated transformation of TALEN construct into *Bt*-black ash genome
  - Gene sequencing for confirming mutation within *AGAMOUS*

References

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Riechmann, J.L., T. Ito, and E.M. Meyerowitz. 1999. Non-AUG initiation of *AGAMOUS* mRNA translation in *Arabidopsis thaliana*. Mol. Cell. Biol. 19:8505-8512.

Acknowledgement

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