Genetically Engineering Black Walnut for Tolerance to Arsenal®

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Abstract

The objective of this study is to engineer black walnut (*Juglans nigra* L.) for herbicide tolerance through an *Agrobacterium*-mediated transformation and regeneration system. We are assembling a genetic construct that will contain a mutated version of the acetohydroxyacid synthase (*AHAS*) gene, which imparts tolerance to the active ingredient in Arsenal®. This herbicide is an imidazolinone and offers excellent pre- and post-emergent control of weeds and competing hardwoods, while providing long-lasting control of competing vegetation between applications. The active ingredient of Arsenal® (imazapyr) will be used as a selection agent in the culture media after transformation. A dose-response curve was generated to determine the effective concentration of imazapyr for selection. Untransformed somatic embryos from two lines of walnut (21 and 91) were used to test five concentrations of imazapyr and 1 µM (0.26 mgL^{-1}) was found to be most effective. The regeneration system is being modified to achieve higher recovery rates. In a maturation and germination experiment, untransformed embryos from four lines (21, 83, 89, and 91) that were initiated from separate seeds from two different trees were used to test the effect of cold treatment (0, 8, 12, 16, and 20 weeks after maturation) on the rate of germination. The ratio of embryos with both a large shoot and root germination increases from 2.5% to 25% as the length of cold treatment increases from zero to 20 weeks. Compared to full-strength germination medium, half-strength germination medium was found to have an influence on the regeneration process. Experiments are underway to improve successful acclimatization of germinated embryos either by transferring them directly to soil, or by multiplying as shoot cultures before rooting.

Fig. 1 (A) Black walnut tree (B) Black walnut cabinet (C) Herbicide spraying for weed control (D) Black walnut secondary embryos

Introduction

Black walnut (*Juglans nigra* L.) is among the most valuable hardwood tree species world-wide, but increasing market demand may soon outstrip our production capacity. A minimum of three years of weed control is needed before black walnut can become established and are free to grow. Currently, weed control is accomplished through mechanical (cultivation) and chemical (herbicides) means. Given the minimum seedling quality standards, herbicide use is the single most important factor for increasing plantation establishment success. However, herbicide treatment options are limited during the growing season because crop trees and competing vegetation are both readily killed by herbicides.

The walnut genome does not contain genes that encode herbicide tolerance; therefore, herbicide-tolerance cannot be introduced via conventional breeding. However, through *Agrobacterium*-mediated transformation, it is possible to introduce the desired trait into the black walnut genome.

Arsenal® is an imidazolinone herbicide which offers excellent pre- and post-emergent control of weeds and competing hardwoods, while providing long-lasting control of competing vegetation between applications because of residual herbicide bound in the soil. Through *Agrobacterium*-mediated transformation, a mutated version of the acetohydroxyacid synthase (*AHAS*) gene could be introduced into the black walnut genome to impart tolerance to Arsenal®. Trees containing the *AHAS* gene could then be over-sprayed with Arsenal® during periods of active growth.

References

Kimura, R., Mandrell, R.E., Galland, J.C., Hyatt, D., and Riley L.W. 2000. Restriction-site-specific PCR as a rapid test to detect enterohemorrhagic *Escherichia coli* O157:H7 strains in environmental samples. Applied and Environmental Microbiology 66(6): 2513–2519.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98: 503-517.

Materials and Methods

Construct Assembly

We will use the pART27 backbone which contains the *nptII* gene (kanamycin resistance), pAC321 which contains an *AHAS* transcriptional unit (in which *AHAS* is driven by a native promoter), and pBI121 which contains the uidA gene (GUS) to assemble a vector for transforming black walnut. Assembled plasmids will be confirmed by restriction analysis and sequencing. The confirmed plasmid will be transformed into a strain of *Agrobacterium tumefaciens* containing a helper plasmid, which provides the *vir* functions.

Transformation and Regeneration

We will use immature somatic embryos as starting material and regenerate them in vitro to prepare embryos for transformation. In a maturation and germination experiment, untransformed embryos from four lines (21, 83, 89, and 91) that were initiated from separate seeds from two different trees were used to test the effect of cold treatment (0, 8, 12, 16, and 20 weeks after maturation) on the rate of germination. The germination and maturation experiment was conducted as follows: (1) convert globular black walnut embryos into cotyledonary embryos; (2) desiccate cotyledonary embryos; (3) desiccated embryos to cold treatment (4℃ in dark for 0, 8, 12, 16, or 20 weeks); (4) embryos to germination medium; (5) embryos to shoot-induction medium; (6) embryos with adequate shoot and root development in soil (acclimatization) or multiply shoot cultures before rooting them.

Imazapyr is the active ingredient in Arsenal® and it has never been used as a selection agent in black walnut in vitro culture. A dose-response test was conducted to determine the effective concentration for selection. Three replications were carried out following the same procedure. For each replication, two lines (21 and 91) were tested for five concentrations (0.01, 0.1, 1, 10, and 100 µM), along with a control. Embryo weight change and secondary embryo production were recorded and analyzed. Using the improved regeneration system, 25 independent transgenic lines will be produced in each of two black walnut genotypes.

Confirm Transgene Insertion and Copy Number

We will verify transgene insertion via PCR. Genomic DNA will be extracted from regenerated lines using a commercial kit (Qiagen) and a buffer that has been optimized for black walnut. PCR products will be amplified from genomic template using primers specific for *AHAS* and *nptII* (Kimura et al., 2000). We will verify copy number using Southern blots (Southern, 1975).

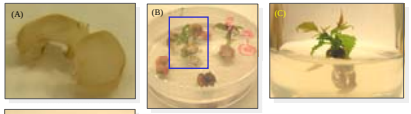
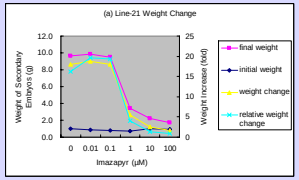
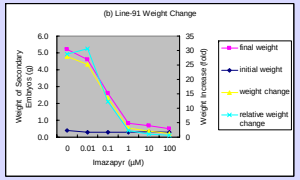
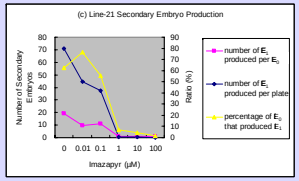


Fig. 2 Maturation and regeneration process
(A) Cotyledonary embryo before maturation
(B) Embryo on germination medium after cold treatment
(C) Embryo on shoot induction medium
(D) Embryo on shoot culture medium
(E) Acclimatization of plantlet







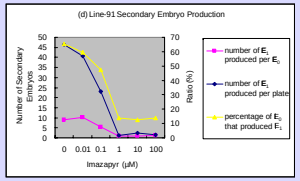


Fig. 5 Imazapyr dose-response test. The effect of five concentrations of imazapyr (control included) on somatic embryos from two black walnut lines, with four Petri plates per concentration per line and six embryos per plate were tested in three replications. Embryo weight change was evaluated in line 21 (**a**) and line 91 (**b**). Embryos on the same plate were weighed together at the beginning and end of the experiment. The following formulas were used: WC=(FW-IW), and RWC=(WC/IW). Number of secondary embryos (E_1) were recorded for each primary embryo (E_0) for line 21 (**c**) and line 91 (**d**), and the mean was calculated across all replicates. Abbreviations: E_0, primary embryo (cotyledonary embryos); E_1, secondary embryo (embryos produced by primary embryos); IW, initial weight; FW, final weight; WC, weight change; RWC, relative weight change.

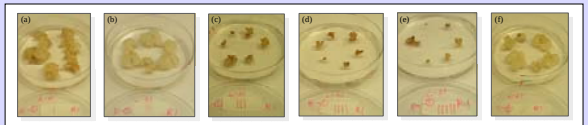
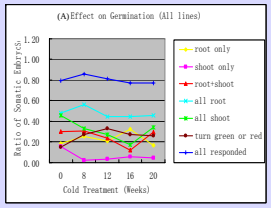


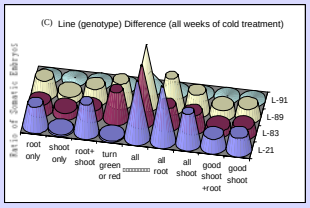
Fig. 4 Embryos in dose-response test. Embryos are from line 21, replication 1. **a-e** are 0.01, 0.1, 1, 10, 100 µM imazapyr, respectively. **f** is the control (without imazapyr).

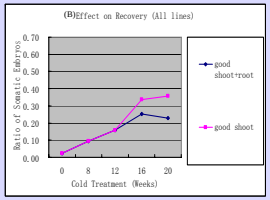
Results and Discussion (Maturation and Germination)

The goal of the maturation and germination experiment was to optimize the regeneration protocol. Figure 2 shows the development from a black walnut somatic embryo to a small plant in the soil.

In Figure 3, (A) did not show strong influence of cold treatment to germination efficiency; (B) indicated an increase in recovery efficiency in terms of increased shoot development (with or without root) after germination; (C) shows that different lines respond differently to the same treatment, and the maturation and germination process is strongly genotype-dependent. Line 21 did best and Line 91 performed poorly; (D) shows that half-strength germination medium is more favorable than full-strength germination medium for the germination and regeneration process.







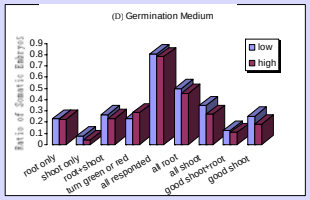


Fig. 3 Maturation and regeneration data analysis (A) Effect of cold treatment on germination. Data are pooled for four lines (21, 83, 89, and 91). (B) Effect of cold treatment on recovery of a plantlet. Data are pooled for four lines (21, 83, 89, and 91). (C) Response of different genotypes to germination and regeneration process. Data are for 0-20 weeks cold treatment. (D) The influence of germination media on the germination and regeneration process. The low is half-strength germination medium and the high is full-strength germination medium. Data are pooled for four lines (21, 83, 89, and 91) and 0-20 weeks cold treatment. All data are the ratios of the number of embryos responding to the initial number of embryos. Embryo response: root only: embryos with only root development during germination; shoot only: embryos with only shoot development during germination; root+shoot: embryos with both root and shoot development during germination; turn green or red: embryos turned green or red but did not develop a root or shoot during germination; all responded: all embryos that responded during germination (the sum of embryos showing one or all of the following: root or shoot development, or turned green or red); all root: all embryos that developed a root; all shoot: all embryos that developed a shoot; good shoot+root: embryos with good shoot and root development after germination; good shoot: embryos with good shoot development after germination.

Results and Discussion (Imazapyr Dose-Response Curve)

Figure 4 are examples of embryos grown under different imazapyr concentrations. We wanted to select a concentration which can effectively prevent secondary embryo production without completely killing the primary embryo, so that we have the best chance to recover transgenic black walnut plants. In this experiment we found 1 µM imazapyr to be most effective.