Kanamycin Sensitivity and Genetic Transformation of Black Walnut Somatic Embryos

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Abstract

Black walnut (*Juglans nigra*) is one of the most valuable hardwoods in U.S. Genetic Improvement of walnut through conventional breeding requires a long-term effort due to its highly heterozygous character and long generation intervals. Genetic engineering is a promising method for tree improvement because specific genetic changes can be made in a short time period and transgenic plants can exhibit the needed traits without loss of genetic integrity. An *Agrobacterium*- mediated gene transfer system that targets cells of English walnut (*Juglans regia*) somatic embryos has been previously reported (McGranahan et al., 1988), but there are no published reports using black walnut. In previous transformation studies with English walnut embryos, transformation efficiency has been very low as result of regeneration of chimeric tissue. For selection of transformed tissue, antibiotics are used in the growth media because transformed tissue harbors a gene for kanamycin resistance. To optimize the dose of kanamycin in selection media, we observed response of untransformed embryos to different concentrations of kanamycin in the media. We found that a range of 100 to 200 mg/L kanamycin was an effective concentration for selection of transformed tissue. *Agrobacterium*-mediated transformation of black walnut somatic embryos was conducted. A hypervirulent *Agrobacterium* strain with an improved promoter in a binary vector was used to increase transformation efficiency of walnut somatic embryos. A high rate of gene transfer was observed with regeneration of 15 % of all co-cultured embryos, but regeneration frequency was more than doubled for those cultured on media without kanamycin. Most of the secondary embryos appeared non transgenic. Histochemical GUS expression assay revealed that less than 10% initial secondary embryos were completely transgenic. We were able to establish five transgenic lines, facilitated by kanamycin selection of 200 mg/L and precocious proliferation of the initial secondary embryos.

Introduction

Genetic improvement of black walnut will help meet the increasing demand for this hardwood species. Long generation intervals and a limit to the potential for genetic gain makes conventional breeding methods expensive and time consuming. Genetic engineering by *Agrobacterium*-mediated gene transfer system of somatic embryos make it an optimal system for faster and efficient system for black walnut improvement.

Objectives

- Optimize kanamycin concentration for efficient selection of transformed embryos.
- Genetic transformation of somatic embryos of black walnut via *Agrobacterium*.
- Selection of transformed embryos.

Fig.1.Type of embryos used for kanamycin sensitivity and genetic transformation. Inset: Typical embryo (close up)

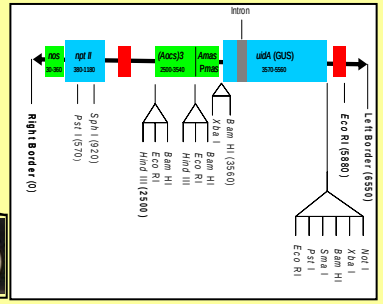

Fig.2. Physical map of pBISN1 T-DNA regions. The T-DNA border sequences are indicated by arrow heads, and colored boxes are used to indicate positions of promoters , open reading frames, and terminators.

Materials and Methods

Kanamycin sensitivity

- 1- 1.5 cm (diameter) somatic embryos of black walnut were used.
- 0, 50, 100, 200, 400, 600, 800,and 1000 mg/L kanamycin were tested.
- Total of 5 lines from two different clones were included in this experiment.
- Growth medium [MS (Murashige and Skoog, 1962), DKW (Driver and Kuniyuki, 1984)] and sucrose concentration (2-3%) varied between lines tested, but all contained 2.4 g/L of agar.
- Non-transformed somatic embryos cultured on growth medium containing different kanamycin levels for 3 months.

Transformation

- *Agrobacterium tumefaciens* strain GV3101 with binary vector pBISN1, β glucuronidase (GUS, *uid*A), and kanamycin resistance (*nptII*) genes were used.
- Embryos were co-cultivated for 60hour with *Agrobacterium* on medium containing acetosyringone(100µM).
- Selection medium contained 500 mg/L Timentin to prevent growth of *Agrobacterium* and 200 mg/L Kanamycin for selection of transformed embryos.
- Secondary embryos produced for three subculture periods were maintained separately on selection medium.
- Labeling system of McGranahan et al.(1988) was employed for embryo generations. Original embryos infected with *Agrobacterium* were labeled as E_o, secondary embryo generation produced on E_o embryos were labeled as E_1,and additional secondary embryo generations produced on E_1 were labeled as E_2.
- Some secondary embryos from each generation were used for histochemical GUS analysis to determine transformants.
- Remaining secondary embryos from the same transformation event were maintained in culture to establish transformed lines.

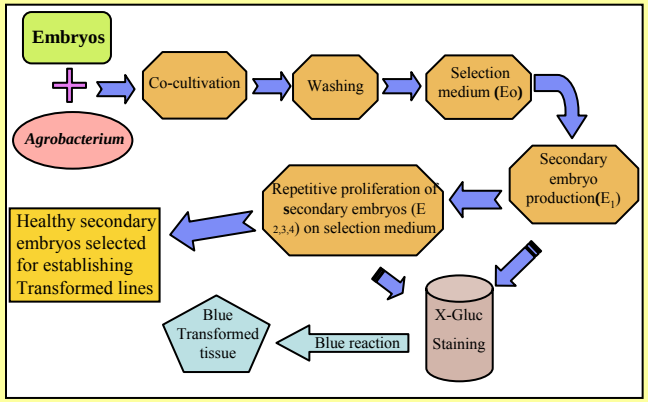

Fig.3. Schematic diagram of protocol for genetic transformation of somatic embryos via *Agrobacterium*.

Results

Kanamycin sensitivity

- Kanamycin drastically effects the growth of somatic embryos (Fig.4).
- Embryo proliferation was inhibited above 400 mg/L kanamycin (Fig. 5).
- Increase in weight and size of embryos was effected most between 50-200 mg/L kanamycin (Fig. 6).
- Secondary embryos produced in the presence of kanamycin exhibited an inhibition of cotyledon differentiation.

Fig.4. Embryos on different kanamycin levels after 3 months.

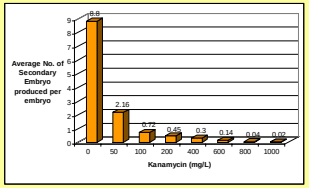

Fig.5. Average number of secondary embryos produced per embryo on medium containing different kanamycin levels. Data combined for all the lines.

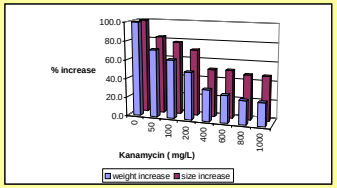

Fig.6. Average percent weight and size increase when embryos were put on medium containing different kanamycin doses.Weight and size increase for medium with no kanamycin is 100%. Data combined for all the lines.

Transformation

- Histochemical GUS expression analysis indicated that the T-DNA transfer rate was high (75-100%).
- Regeneration efficiency was 15 % for the embryos exposed to *Agrobacterium.*
- Most of the embryos that were positive for GUS staining were transgenic, but chimeric (Fig. 8).
- Kanamycin concentration of 200 mg/L was effective for selection of transgenic lines.
- Less than 10 % embryos of the initial secondary embryos were transgenic (showed complete staining) as assessed by GUS analysis.

Table 1. Growth and proliferation responses of E_1[a] embryos

Treatment	Media	N	Growth[b] (%)	Proliferation[c] (%)	Production stable lines[d] (%)
No Selection	Kan	15	0	0	0
	No Kan	35	63	55	0
Kanamycin Selection	Kan	114	7	5	5
	No Kan	34	9	6	0

[a] Secondary embryos produced from E_o or original embryos exposed to *Agrobacterium.*
[b] Percentage of embryos with definite cotyledon enlargement (to at least 5 mm in diameter)
[c] Percentage of embryos producing secondary embryos, typically observed after 2-3 sub-culture periods.
[d] Percentage of embryos giving rise to stable kanamycin-resistant lines.

Table 2. Conformation of transformed tissue by X-GLUC staining

Treatment	Embryo Type[a]	N	GUS Positive N	GUS Positive %
Kanamycin Selection	E_0	44	44	100
	E_1	95	74	78
	E_2	17	17	100[b]
No Selection	E_0	38	28	74
	E_1	68	11	16
	E_2	6	0	0

[a] Original embryos infected with *Agrobacterium* were labeled as E_o. Secondary embryo generations produced on E_o embryos were labeled as E_1 and further secondary embryo generations produced on E_1 were labeled as E_2. The E_1 and E_2 embryos are distinguished based upon whether they were stained directly or after one or more months of individual culture. Those that were subcultured are further distinguished based upon whether they exhibited a positive growth response.
[b] All of the E_2 embryos evaluated were derived from the same two transformation events.

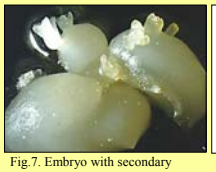

Fig.7. Embryo with secondary embryos.

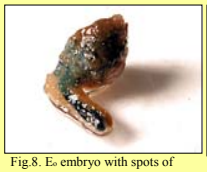

Fig.8. E_o embryo with spots of GUS positive cells.

Fig.9. Positively stained embryo.

Conclusions

- Kanamycin has a significant effect on growth and secondary proliferation of black walnut somatic embryos.
- Kanamycin concentration of 100-200 mg/L is suitable for use in selection medium.
- Black walnut somatic embryos can be transformed via *Agrobacterium*-mediated transformation.
- Transformation frequency was lower than expected, but can be further increased by using smaller size embryos and decreasing the kanamycin concentration to allow more secondary embryo proliferation on selection medium.

References

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