Kanamycin Sensitivity and Genetic Transformation of Black Walnut Somatic Embryos

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Abstract:

Genetic engineering is a promising method for tree improvement because specific genetic changes can be made in a short period of time and transgenic plants can exhibit these traits without loss of genetic integrity. An *Agrobacterium*-mediated gene transfer system that targets somatic cells of walnut has been previously reported, but transformation efficiency was low and resulted in the generation of chimeric tissue. Results from previous experiments showed a gene transfer rate of 75-100% using the binary vectors pBISN1 and pBI121. Although gene transfer was detected in nine out of ten genotypes of black walnut somatic embryos, secondary embryo production was observed in only 15% of the infected embryos. Five percent of all secondary embryos produced three transgenic lines. The objective of this study was to improve transformation efficiency. We used untransformed embryos from five lines (21, 28, 83, 89, and 91) that were initiated from five different fruits from two different trees. We tested kanamycin at 0, 50, 100, 200, 400, 600, 800, or 1000 mgl^{-1} and found 100 mgl^{-1} kanamycin in the medium was effective for efficient selection and while maintaining production of secondary embryos. *Agrobacterium*-mediated transformation of black walnut somatic embryos was conducted using the binary vector pBISN1 containing the *npt*II and *uid*A (GUS) genes with an intron for prevention of GUS expression in residual bacteria in transformed tissue. A T-DNA transfer rate of 54-100% occurred, varying among the five lines tested. Secondary embryo production (55%) was observed in the infected embryos. Embryos from all five lines were transformed, but only line 91 was able to produce stable transgenic lines as revealed by histochemical GUS assays. After 28 wk on selection medium, 14 completely transformed lines were established by proliferation of secondary embryos. Fifteen percent of the total (E_0) embryos infected in line 91 were able to produce stable transgenic lines. Each transgenic line originated from single secondary (E_1) embryo which originated from seven individually infected parent (E_0) embryos. Each of these transgenic lines were considered to be a separate transformation event. Eleven percent of secondary embryos were established as transgenic lines. These results will be further validated by PCR. Earlier maturation and germination experiments showed 18% plantlet production from untransformed embryos. Embryos from these transgenic lines are successfully regenerating new somatic embryos in vitro with a similar maturation and germination efficiency (21%) in medium containing kanamycin. A more efficient transformation system for black walnut embryos which used a lower selection pressure (100 mgl^{-1} kanamycin) but for a longer duration (28 wk) and the continuation of selection during repetitive somatic embryo production improved the transformation system for black walnut.

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 of somatic embryos will be a faster and efficient system for black walnut improvement.

Objectives:

- Determine the effective concentration of kanamycin to select transformed tissue.
- Genetic transformation of somatic embryos of black walnut.
- Selection of transformed embryos.
- Germination of transformed embryos.

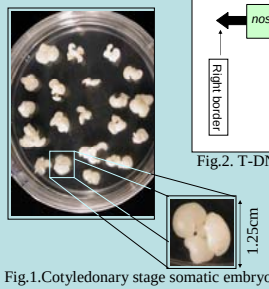

Fig.1.Cotyledonary stage somatic embryos. Inset: Close up of an embryo

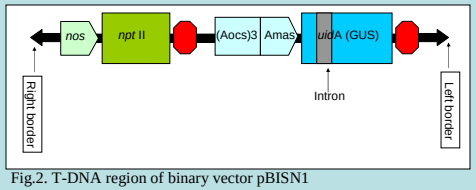

Fig.2. T-DNA region of binary vector pBISN1

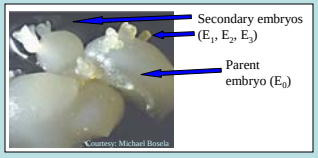

Fig.3. Secondary embryo production on cotyledon surface of an embryo

Materials and Methods:

Kanamycin Sensitivity:

- Cotyledonary stage (1- 1.5 cm in diameter) black walnut somatic embryos were used.
- Somatic embryos from five lines (line 21 and 91 from tree 352; line 28, 83, and 89 from tree 378). Each line was initiated from a single fruit.
- Embryos were cultured on DKW (1) or MS (2) medium containing kanamycin at 0, 50, 100, 200, 400, 600, 800, or 1000 mgl^{-1} for 12 wk.

Transformation:

- Cotyledonary stage somatic embryos from five lines (21, 28, 83, 89, and 91) were used.
- *A. tumefaciens* strain GV3101/pMP90 was used with binary vector pBISN1 having *uid*A (GUS) (with intron) as marker gene and *nptII* gene as selectable gene.
- Embryos were co-cultivated with *Agrobacterium* on DKW or MS medium containing 100µM acetosyringone, then transferred (DKW or MS) medium containing 500 mgl^{-1} timentin to prevent growth of *Agrobacterium* and 100 mgl^{-1} of kanamycin for selection of transformed embryos for 11 wk.
- Original embryos infected with *Agrobacterium* were labeled as E_0, secondary embryos produced on E_0 were labeled as E_1 and subsequent generations of secondary embryos produced were labeled as E_2, E_3, E_4, etc.
- E_1 embryos were harvested and placed on selection medium before staining E_0 embryos at end of 11 wk.
- Secondary embryos produced from each generation were used for histochemical GUS assays, to determine transformants.
- Secondary embryos from the same transformation event as those staining positive for GUS assays were maintained in culture to establish transformed lines.
- Subsequent generations of secondary embryos were evaluated for GUS up to 28 wk.

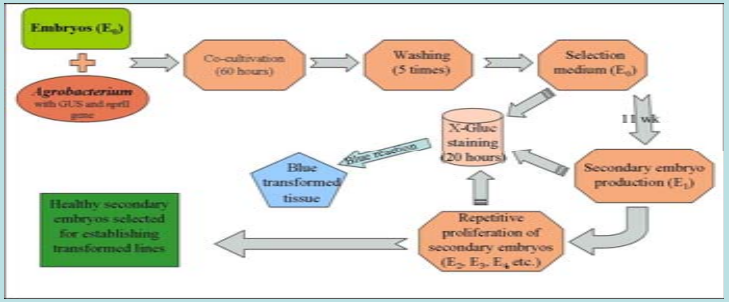

Fig.4. Schematic diagram of different steps in genetic transformation of somatic embryos via *Agrobacterium.*

Maturation and Germination:

- Cotyledonary stage embryos from five lines were used.
- Embryos matured by desiccation for 3 wk in the dark in 97% relative humidity (RH).
- Embryos germinated on DKW basal medium for 4 wk in 16-h photoperiod (55 µmol m^{-2} s^{-1}).
- Shoot development on DKW medium with 2.5 µM zeatin for 4 wk in 16-h photoperiod (55 µmol m^{-2} s^{-1}).
- Acclimatization of germinated embryos in Scotts® 366P soil mixture and gradual reduction of RH.

Results:

Kanamycin Sensitivity:

- Kanamycin drastically effects the growth of somatic embryos (Fig.5).
- Embryo proliferation was reduced at 100 mgl^{-1} kanamycin (Fig. 6).
- Increase in weight and size of embryos was effected most between 50-200 mgl^{-1} kanamycin (Fig. 7).
- Secondary embryos produced in the presence of kanamycin exhibited an inhibition of cotyledon differentiation.

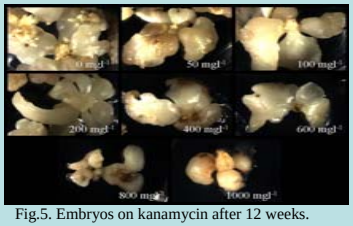

Fig.5. Embryos on kanamycin after 12 weeks.

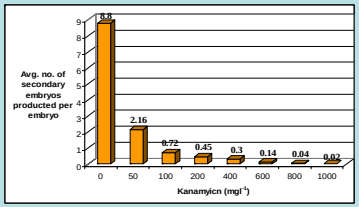

Fig.6. Average number of secondary embryos produced per embryo on medium containing kanamycin. Data combined for all five lines.

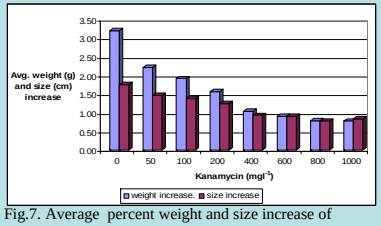

Fig.7. Average percent weight and size increase of embryos on medium containing kanamycin. Data combined for all five lines.

Results:

Transformation:

- Gene transfer was successful for all five lines with a frequency of 54-100% (Fig. 8a).
- Fifty five percent of all infected (E_0) embryos produced secondary (E_1) embryos.
- Larger proportions of tissue showing positive GUS expression with each next generation (Fig. 8).
- Completely transformed embryos from line 91.
- For line 91, after 28 wk on selection medium, 15% (7/48) of infected (E_0) embryos produced 14 stable completely transgenic lines.
- Each transformed line was established from a single secondary (E_1) embryo.
- Ninety eight percent (41/42) of secondary embryos (E_3) were completely transformed as revealed from GUS assays. (Table 1 and Fig. 8d)
- Eleven percent (14/123) of all secondary embryos produced for line 91 established stable transgenic lines.

Table 1: GUS positive expression in different embryo generations

Line [1]	Control [2]	E_0		E_1		E_2		E_3	
		No.	%[3]	No.	%	No.	%	No.	%
21	0	45	94	17	29	3	14	0	0
28	0	29	60	4	15	0	0	0	0
83	0	35	73	5	14	0	0	0	0
89	0	26	54	0	0	0	0	0	0
91	0	48	100	44	36	30	33	41	98

[1] Data combined from three replications.
[2] Embryos were treated the same as the infected embryos except no contact with *Agrobacterium* at any stage.
[3] Percent of total embryos stained showing positive GUS expression.

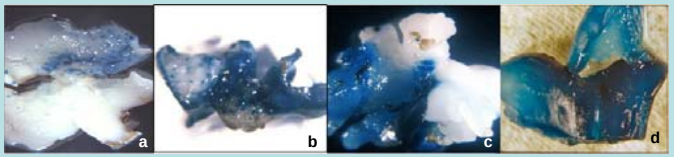

Fig.8. Different degrees of positive GUS expression seen in transformed embryos.

Maturation and Germination :

- Transformed and untransformed embryos matured and germinated.
- High proportion of embryos germinate with only roots (64%) (Fig. 9a)
- Embryos develop roots only on germination medium but shoot development was observed in 18% of all embryos matured (Fig. 9b&c).
- Acclimatization of plantlets is currently underway (Fig. 9d).

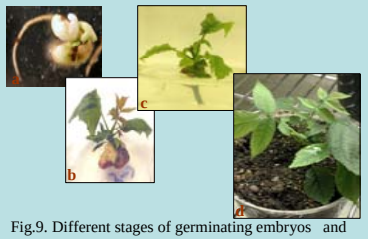

Fig.9. Different stages of germinating embryos and acclimatized plants.

Discussion:

- Black walnut somatic embryos can be transformed by *Agrobacterium*- mediated transformation.
- Transformation efficiency is genotype dependent as only one line (91) out of five lines tested was able to produce completely transformed lines as revealed by histochemical GUS assays.
- Kanamycin concentration of 100 mgl^{-1} is suitable for use in selection medium.
- Previous experiments (3) had revealed a similar gene transfer rate (75-100%), but transformation efficiency was lower (5%) at kanamycin concentrations of 200-500 mgl^{-1}.
- Transformation efficiency can be increased by selection at lower selection pressure and at advanced generations (E_3 or E_4).
- Further improvement of this transformation system may be achieved by using more virulent strains of *Agrobacterium* and early cotyledonary stage somatic embryos.
- Maturation and germination studies of transformed black walnut embryos have shown good germination percentages (18%).

References:

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