

# GENETIC TRANSFORMATION OF BLACK WALNUT (*JUGLANS NIGRA*)

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**ABSTRACT**—Disarmed *Agrobacterium tumefaciens* strains with binary vectors carrying transgenes for kanamycin resistance (*npt II*) and  $\beta$ -glucuronidase (GUS, *uidA*) were used for the genetic transformation of Eastern black walnut (*Juglans nigra*) somatic embryos. In total, explants from 16 embryo lines, representing 10 genotypes, were used and regeneration was compared both in the absence of kanamycin selection and with different levels of selection stringency (200-250 or 500 mg/L kanamycin). Histochemical GUS expression assays indicated that the rates of T-DNA transfer were high (75-100%), with gene transfer being demonstrated for 14/16 lines. Regeneration was observed for 15% of the infected embryos, compared with frequencies of 50% for the non-infected controls. The regeneration frequencies were nearly two-fold higher when kanamycin was excluded from the culture medium, but most of the secondary embryos appeared non-transgenic. When kanamycin was present, most of the secondary embryos were transgenic, but chimeric, consisting of mixtures of transgenic and non-transgenic cells; and few exhibited *de novo* growth following harvest, even on non-selective medium. Less than 10% of the initial secondary embryos were wholly transgenic, as assessed by GUS assay; however, these embryos appeared essential for the initiation of stable transgenic lines. The establishment of transgenic lines was also facilitated by the use of lower kanamycin concentrations (200-250 mg/L) and by the precocious proliferation of the initial secondary embryos.

## INTRODUCTION

In contrast with conventional breeding, which primarily involves gene manipulation at the level of entire chromosomal regions, genetic engineering is more precise. Genetic transformation techniques can be used to alter the expression patterns of individual genes in a predictable manner, with silencing, over-expression, and ectopic expression all being possible. Genes can be transferred across species barriers and genetic transformation protocols can be used for gene discovery.

Genetic transformation is especially attractive in light of the recalcitrance of most trees for conventional breeding. In contrast with agronomic crops, trees are characterized by long sexual generation times, high levels of heterozygosity (Hamrick 2000), and effective progeny screening is hindered by their large size and a frequent lack of juvenile:mature correlations, especially for vegetative characteristics (Ostry and Michler 1993,

Tzfira and others 1998). For trees that are routinely grown in plantations, such as black walnut, the introduction genes for herbicide tolerance or pest resistance would be desirable. In addition, over a longer timeframe, strategies for the manipulation of wood quality traits (increased heartwood content, etc.) could be developed.

We are not aware of any previous publications describing the genetic transformation of black walnut; however, the genetic engineering of English walnut (*Juglans regia*), using disarmed strains of *Agrobacterium tumefaciens*, has been widely reported. In fact, *J. regia* was the first plant species (woody or non-woody) to be transformed using somatic embryos (McGranahan and others 1988, Dandekar and others 1989). Although the initial transformation frequencies were low (3%), the authors were able to establish that physical wounding was not necessary for gene transfer and that transformation competence was related to the size of the embryos. The efficacy of the

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protocol was improved via the use of selective media (McGranahan and others 1990); however, the initial secondary embryos regenerated were frequently chimeric (i.e., consisting of mixtures of transgenic and non-transgenic cells) or entirely non-transgenic, even when selective media was used (McGranahan and others 1990, Escobar and others 2000). Derivative protocols have been developed in several additional labs (El Euch and others 1998, Tang and others 2000); however, in all cases, the development of efficient selection protocols has proved challenging.

In this research, we used the basic *J. regia* transformation protocol as a model for the development of initial *J. nigra* transformation protocols. However, our research differed from that of our predecessors in that media with multiple kanamycin concentrations were evaluated for selection. In addition, to increase the likelihood of concurrent secondary embryo induction and gene transfer, the embryos used for inoculation were larger (more mature) than those that have typically been used by previous experimenters.

## MATERIALS AND METHODS

### Plant Material

Somatic embryo cultures of *J. nigra* were induced from the cotyledon explants of immature zygotic embryos during the summer of 1999 (Bosela and Michler 2000). Walnut fruits were collected at 2-3 week intervals from mid-July to the end of August for culture initiation. In total more than 40 embryo lines (differing in genotype or induction media) were obtained, including nearly 20 lines that have been maintained continuously in vitro to date on a 3-5 week transfer cycle. The lines have been cultured primarily on basal (hormone-free) media; however the specific media types (MS or DKW) and sucrose concentrations (20 or 30 g/L) used have varied between the embryo lines.

### Somatic Embryo Proliferation

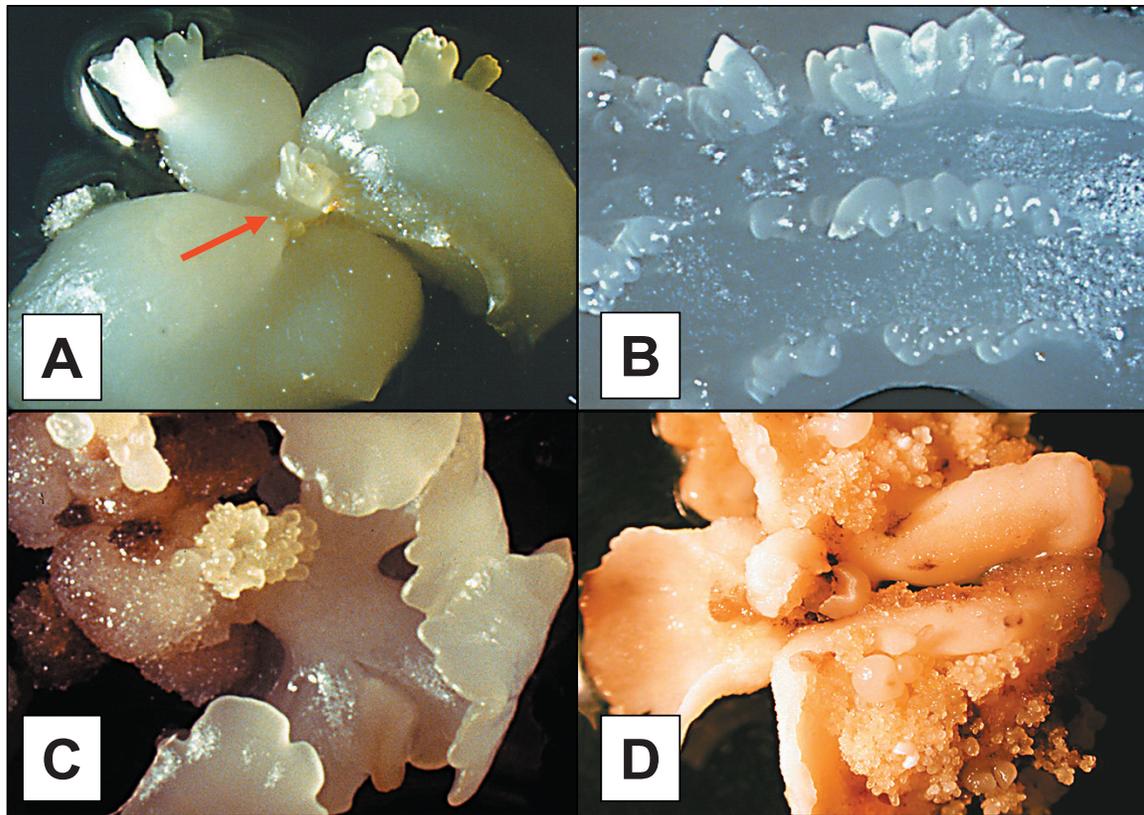
Two mechanisms of proliferation were recognized for the lines, which are termed direct and indirect here, based on the degree of connectivity between the parent embryos and secondary embryos. Direct proliferation is characterized by the production of secondary embryos that are physically attached to the parent embryo (Fig. 1A). Most are produced from the cotyledons and are frequently arranged in rows that may correspond with the position of subtending vascular tissues (Fig. 1B). Cotyledon

differentiation is typically observed shortly after embryo emergence (Fig. 1B), and continued proliferation is achieved via the subculture of the initial secondary embryos. However, since proliferation competence and embryo size are positively correlated, one to two culture periods of growth are typically required prior to the production of additional embryo generations.

Indirect proliferation is characterized by the production of secondary embryos that proliferate precociously (i.e., prior to cotyledon differentiation) to form masses of globular embryos, which lack obvious direct connections with the parent embryo (Figs. 1C, 1D). On basal medium, the degree of globular embryo proliferation is typically limited, with most enlarging and differentiating as cotyledonary embryos within 1-2 culture periods of their initiation. However, the continuous proliferation of globular embryos is generally possible upon transfer to MS medium with 2,4-D (0.2 – 2  $\mu$ M). For the cumulative data set, indirect proliferation was only erratically observed; however, for some embryo lines, greater numbers of embryos were produced via indirect proliferation than direct proliferation. In addition, indirect proliferation appears to be facilitated by the use of MS media, elevated sucrose concentrations (30-50 g/L), and the inclusion of organic nitrogen sources (casein hydrolysate and glutamine).

### *Agrobacterium* Strains

The GV3101/pMP90 strain of *Agrobacterium tumefaciens* (Koncz and Shell 1986) was used in conjunction with two binary vectors; pBI121 (Jefferson and others 1987) and pBISN1 (Narasimhulu and others 1996), depending upon the experiment. pBI121 and pBISN1 are nearly isogenic. Both are derived from pBIN19 (Bevan 1984) and contain a kanamycin resistance gene (*npt II*) on the plasmid backbone. In addition, the T-DNA (transferred DNA) regions are equivalent aside from differences in  $\beta$ -glucuronidase (*uidA*) gene construction (Fig. 2). In pBI121, the *uidA* gene is regulated by the CaMV 35S promoter (Guilley and others 1982), while in pBISN1 it is combined with the “super promoter” of Dr. Stanton Gelvin’s lab (Purdue University, West Lafayette, IN, USA). The super promoter consists of a trimer from the octopine synthase promoter’s upstream activator sequence and a single copy of the mannopine synthase promoter and its upstream activator (Ni and others 1995, Fig. 2). Although both promoters have been generally observed to be “strong” and constitutive in their expression patterns, in tobacco leaves the levels of GUS expression achieved using the super promoter have been shown to be up to 156-fold greater than the equivalent gene



**Figure 1.—Embryo proliferation mechanisms. (A)** A larger embryo with direct secondary embryos from two of its three cotyledons. The epicotyl of the embryo is indicated with an arrow. **(B)** A cotyledon with rows of secondary embryos of direct origin. The largest secondary embryos shown are only 0.5-1 mm in size yet most have already differentiated cotyledons. **(C)** A cotyledonary embryo with a sheath of gelatinous callus and an associated mass of globular to polar embryos from its hypocotyl-root axis. **(D)** A larger embryo with masses of globular to polar embryos of indirect origin associated with its cotyledons.

expression levels observed for the CaMV 35S promoter (Ni and others 1995). The *uidA* gene of pBISN1 also differs from that of pBI121 in having an intron in its open-reading frame that precludes expression in *Agrobacterium* (Vancanneyt and others 1990, Liu and others 1992).

## Transformation

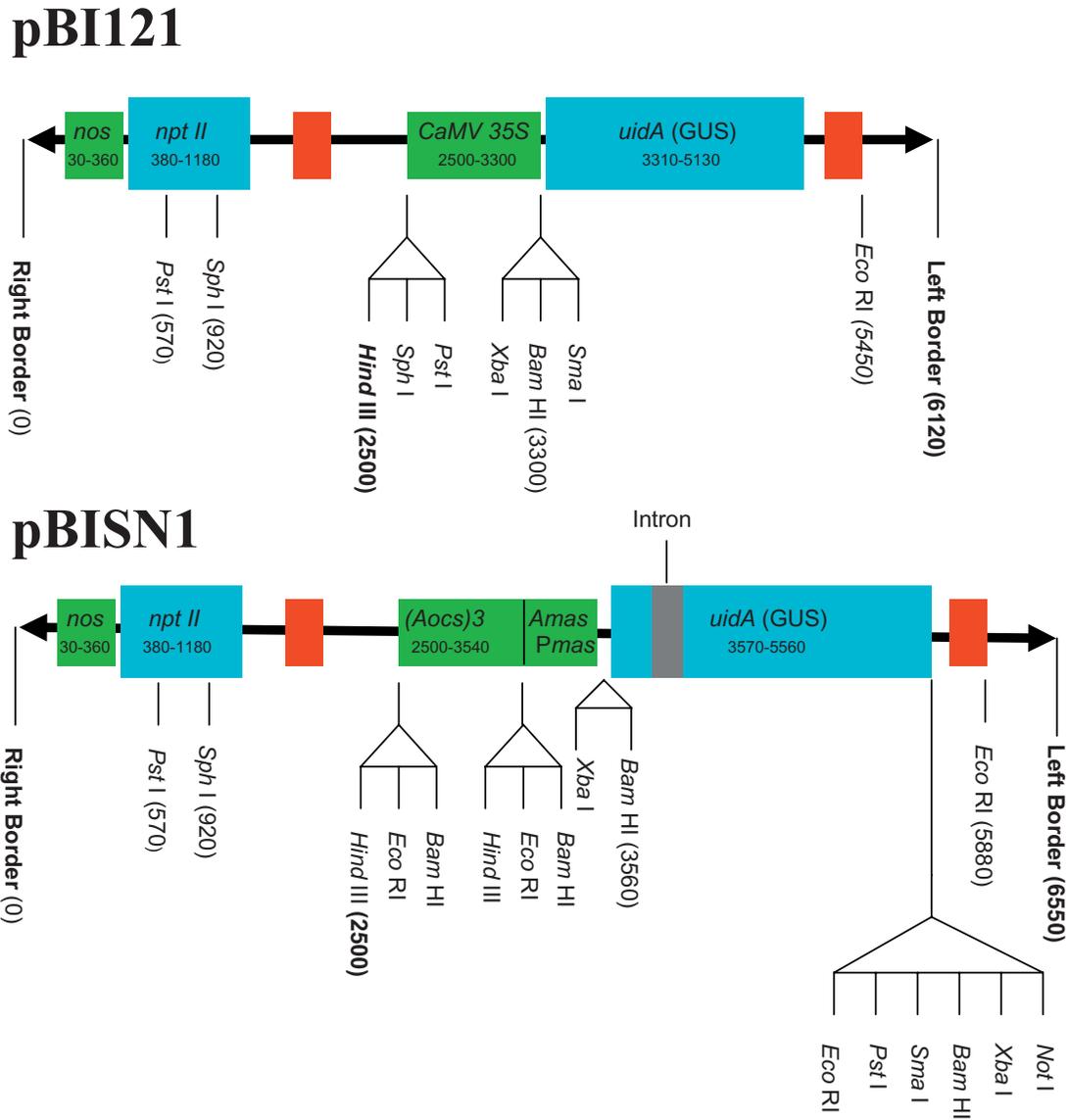
### Explants

In total, somatic embryos from the 16 distinct embryo lines, representing 10 distinct genotypes, were used. In general, the inoculated embryos were of direct origin, but embryos of indirect origin were also used. In either case, the embryos were generally isolated at an early cotyledonary stage of development and cultured individually for 3-4 weeks on basal medium prior to inoculation. The infected embryos averaged 7-10 mm in size, but some were significantly larger (up to 20-30 mm in diameter), and were frequently broken either inadvertently during disinfection or intentionally

(prior to co-cultivation) to facilitate contact between the explants and antibiotics in the media.

### Bacteria Cultures

The *Agrobacterium* cultures were grown in Luria-Bertani (LB) media [10% (w/v) tryptone, 5% (w/v) yeast extract, 10% (w/v) NaCl, pH 7.0] on an orbital shaker at 28° C at 200 rpm. The initial cultures were prepared in 3 mL volumes and 0.5-1 mL aliquots from these cultures were used to seed larger cultures (25-50 mL) that were used for infection. Gentamycin (25 mg/L) and kanamycin (50 mg/L) were used to select for the disarmed Ti plasmid (pMP90) and binary vectors, pBI121 and pBISN1, respectively. The LB media used for the initial (3 mL) cultures, was also spiked with rifampicin (40 mg/L) to preclude *Escherichia coli* contamination. Once the cultures had grown to an absorbance at 600 nm ( $A_{600}$ ) of 0.3-0.9, they were pelleted by centrifugation (1000xg, 10-15 min) and re-suspended in antibiotic-free media at a density of approximately  $\approx 5 \times 10^8$  cells/mL (Leple and others 1992, Han and others 1996).



**Figure 2.—Physical maps of the pBI121 and pBISN1 T-DNA regions. The T-DNA border sequences are indicated with arrowheads, and colored boxes are used to indicate the positions of promoters, open reading frames, and terminators. Green boxes indicate promoter elements; blue boxes indicate open reading frames; and red boxes indicate transcription terminator sequences. Map positions ( $\pm 10$  bps) are indicated relative to the position of the right T-DNA border sequences. The cutting sites for several common restriction enzymes are also shown. Note: In pBISN1, the uidA promoter is chimeric, consisting of a trimer upstream activator sequences from the octopine synthase (ocs) promoter and a single copy of the mannopine synthase (mas) promoter and upstream activator (see Materials and Methods).**

For the 2002 experiments ( $n = 3$ ), LB medium was used for *Agrobacterium* re-suspension. In contrast, the cultures from the 2003 experiment were resuspended in liquid plant culture media (MS vs DKW). Acetosyringone (20  $\mu$ M) was added and the cultures were grown for an additional 20-30 min for virulence gene induction.

**Infection, Co-cultivation, and Disinfection**

For infection, the somatic embryos were divided into sterile 50 mL centrifuge tubes with aliquots (3-5 mL) from the virulence-induced *Agrobacterium* cultures and placed on a rotary shaker at room temperature ( $23 \pm 2^\circ$  C). After 20-30 min the embryos were removed, blotted dry, and

transferred to plates of basal medium with 100  $\mu$ M acetosyringone in the dark for co-cultivation. The embryos were removed from the co-cultivation plates after an additional 50-65 hr and “washed” five to six times, with the final three washes containing antibiotic (500 mg/L cefotaxime for the 2002 experiments or 500 mg/L timentin for the 2003 experiment) to prevent the growth of residual *Agrobacterium* cells. The explants were washed in sterile 50 mL centrifuge tubes on a rotary shaker with 15-20 min/wash. The medium used for washing (sterile water or liquid plant culture media) varied between experiments.

### Regeneration

Following washing, the embryos were blotted dry on sterile paper towels and transferred to plates of basal medium containing the same antibiotics as had been used for washing, (i.e., 500 mg/L cefotaxime or 500 mg/L timentin). For each embryo source, the embryos were divided between selective and non-selective media. In general, only 4-6 embryos were assigned to the non-selective medium, and the rest were assigned to selective medium. For most embryo sources, only a single replicate of each treatment was prepared; however, when enough explants were present, multiple replicates of the kanamycin treatments were prepared (5-10 embryos per replication).

The explants were transferred to fresh media every 3-4 weeks. Embryos assigned to the selective media were maintained for three culture periods, while those assigned to non-selective media (and the controls) were maintained for only two culture periods. Secondary embryos were harvested at the end of the same culture period when they first became apparent and sorted into transformation events based on their spacing (i.e., embryos found within 5 mm of one another were considered to be derived from the same transformation event, while embryos spaced further apart were presumed to represent separate transformation events). Depending on the number of transformation events and embryos per transformation event, the secondary embryos were subcultured (to one or more media types), stained for GUS activity, or both.

The labeling system of McGranahan and others (1988) was used to distinguish between embryo generations (i.e., the inoculated explants were designated as  $E_0$  embryos, the initial secondary embryos were termed  $E_1$  embryos, and the subsequent generations of embryos were assigned progressively higher order subscripts [ $E_2$ ,  $E_3$ , etc.]). Proliferation was generally direct with the embryo generations being separated by 1-2 culture periods of growth. However, when the initial secondary

embryos proliferated indirectly (at the globular stage), embryo generations were impossible to distinguish.

### Selection

For the 2002 experiments, media with lower (250 mg/L) and higher (500 mg/L) kanamycin concentrations were tested in separate experiments. However, for the 2003 experiment, the effects of differences in kanamycin concentration were evaluated more directly. The basic selective medium contained 500 mg/L kanamycin; however, for lines with sufficient numbers of embryos for the preparation of three more replicates, the embryos were divided between media with 500 mg/L kanamycin (2 replicates) and 200 mg/L kanamycin (single replicates). The 200 and 500 mg/L kanamycin media were also compared using  $E_1$  and  $E_2$  embryos, especially when multiple embryos were available from the same transformation events.

The sensitivity of non-transgenic walnut somatic embryos to kanamycin was evaluated in a series of preliminary, supplemental experiments. Kanamycin was tested at concentrations of 25, 50, 100, 250, 500, and 1000 mg/L, using both small (2-5 mm) and large (10-20 mm) cotyledonary embryos. Embryos from multiple lines were evaluated; however, replicates of the treatments were not prepared on a per embryo line basis. After 4-6 weeks, the embryos were evaluated for proliferation. The health of the embryos was also assessed and the small cotyledonary embryos were evaluated for *de novo* growth.

### GUS (*uidA*) Expression Assays

Somatic embryos were screened for GUS activity by histochemical assay (Jefferson and others 1987). The staining solution contained 1mM X-Gluc, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1 M sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, and 2% dimethyl sulfoxide (DMSO), as recommended by Stomp (1992). Staining was carried out at 37° C for 16 to 20 h, followed by clearing in 70% ethanol. Samples with blue staining (indigo precipitate) were scored as positive for GUS gene activity.

## RESULTS AND DISCUSSION

### Initial ( $E_1$ ) Regeneration

#### Secondary Embryo Formation

For the 2003 experiment, the regeneration competence of the  $E_0$  embryos was adversely

affected by co-cultivation (Table 1), as has been previously observed for *J. regia* somatic embryos (Tang and others 2000). Similar differences in regeneration frequency between the infected and non-infected embryos were not apparent for the 2002 experiment, presumably as a result of the recalcitrance of the embryos for regeneration in general, with secondary embryo formation being observed for only 15% of the control embryos (Table 1), compared with more typical regeneration frequencies of 40-75% as assessed during culture maintenance (data not shown). Several factors, including differences in binary vector type, antibiotic type, and embryo line representation may have contributed to the contrasting regeneration responses. However, the use of different media for *Agrobacterium* resuspension (i.e., LB medium for the 2002 experiments and plant medium for the

2003 experiments) is thought to have played a critical role.

For the infected embryos, the regeneration frequencies were approximately twice as high when non-selective media was used (Table 1), and the differences were statistically significant ( $\alpha = 0.05$ ) for both data sets (2002 and 2003) as evaluated by Chi-Square analysis. The regeneration frequencies were also highly dependent on the concentration of selective agent, with the frequencies of secondary embryogenesis being two- to three-fold greater when lower (200-250 mg/L) rather than higher kanamycin concentrations (500 mg/L) were used (Table 2). The number of transformation events and secondary embryo yields per responsive explant ( $E_0$  embryo) were also much higher for the 200 mg/L kanamycin treatment (Table 2).

**Table 1.—Regeneration frequency by experimental treatment and calendar year. For each treatment, the data is combined across embryo lines and experimental replicates. The direct, indirect, and cumulative (total) regeneration frequencies are indicated.**

Year <sup>1</sup>	Treatment <sup>2</sup>	N	Regeneration (%)		
			Total	Direct	Indirect
2002	Control (not infected)	20	15	15	0
	No selection	54	14	10	4
	Kanamycin selection	215	8	7	1
2003	Control (not infected)	101	57	54	3
	No selection	85	30	24	6
	Kanamycin selection	346	17	16	1

<sup>1</sup> Experimental conditions varied between years. For the 2002 experiments, the GV3101/pMP90/pBI121 A. tumefaciens strain was employed, the bacteria were resuspended in LB medium prior to infection, and cefotaxime was used for *Agrobacterium* elimination. For the 2003 experiment, a different binary vector (pBISN1) was employed, plant media (MS or DKW) was used for resuspension, and timentin was used for *Agrobacterium* elimination.

<sup>2</sup> The control embryos were subjected to the same infection, co-cultivation, and disinfection regimes as were typical for the experimental treatments; however, “blank” *Agrobacterium* resuspension medium was used for inoculation and antibiotic-free media were used for regeneration.

**Table 2.—Relationship between kanamycin (Kan) concentration and transformation efficacy.**

Year <sup>1</sup>	Kan (mg/L)	% Regeneration	Transformation Events Per Responsive Embryo <sup>2</sup>	Secondary Embryo Yield Per Responsive Embryo <sup>3</sup>
2002	250	18	N.D.	N.D.
	500	5	N.D.	N.D.
2003	200	33	2.9	5.7
	500	13	1.4	1.4

<sup>1</sup> In 2002, the 250 and 500 mg/L kanamycin media were tested in separate experiments, employing different combinations of embryo lines. In 2003, the 200 and 500 mg/L kanamycin media, were tested in a single experiment using explants that were genotypically and physiologically equivalent. Note: The regeneration frequencies presented here are not directly comparable with those shown in Table 1, since the 2003 data presented here is only for the subset of embryo lines that were tested on both types of kanamycin media.

<sup>2</sup> The mean number of transformation events per embryo for the subset of  $E_0$  embryos with regeneration (i.e., secondary embryogenesis). Note: The  $E_1$  embryos from the 2002 experiments were not sorted by transformation event.

<sup>3</sup> Mean yield of secondary embryos per embryo (combined across transformation events, see above) for the subset of  $E_0$  embryos with regeneration, as calculated for the 2003 experiment.

### GUS Expression Assays

To assess the frequency of T-DNA transfer and integration, several  $E_0$  embryos that had remained sterile and relatively healthy, as assessed by their limited degree of discoloration, were stained for GUS activity. For the 2002 experiments nearly 85% (21/25, data not shown) of the  $E_0$  samples tested positive. Since the *uidA* gene used for the 2002 experiments did not contain an intron it is impossible to exclude the possibility that the blue staining may have resulted from T-DNA expression by residual, interstitial *Agrobacterium* cells. However, similarly high staining frequencies were obtained for the 2003 experiment (Table 3).

The preferential staining of only the healthiest  $E_0$  embryos may have biased the staining results, especially if the rates of embryo discoloration and T-DNA transfer were inversely related. However, since the  $E_0$  embryos evaluated in this study were stained primarily at the end of the second or third culture periods, their degree of browning (senescence) was frequently quite advanced, and selection of the least discolored embryos was thought necessary to ensure the visualization of cells exhibiting a positive staining response. In fact, it could be argued that T-DNA transfer rates reported are lower than the staining frequencies that would have been obtained if the embryos had been stained by the end of the

first culture period. However, perhaps even more telling than the high frequencies of GUS-positive staining observed for the cumulative data set, is the consistency with which positive staining results were observed across embryo lines; i.e., stable *uidA* gene integration was demonstrated for 14 of 16 embryo lines evaluated, representing 9 of 10 genotypes.

For most  $E_0$  embryos the staining observed was primarily distributed as discrete blue spots, the smallest of which were estimated to be only 20-25  $\mu\text{m}$  in size, and thought to represent single transgenic cells (Figs. 3A, 3B). However, for some of the  $E_0$  embryos, a subset of the blue spots were distinctly larger (0.2-0.5 mm in diameter) and were presumed to represent clones of daughter cells from the same transformation event (Fig. 3C). In other cases patches or bands of blue were apparent, especially in the vicinity of regions, such as the root axis or cotyledon bases, that would be expected to show rapid and directional growth during embryo development (Fig. 3D). The density of blue foci (spots and patches) was highly variable between embryos (Fig. 3) and appeared to be related to differences in embryo growth rate (i.e., the number of transformation events detected per embryo was greatest for embryo sources that exhibited the most vigorous growth as non-infected controls [data not shown]).

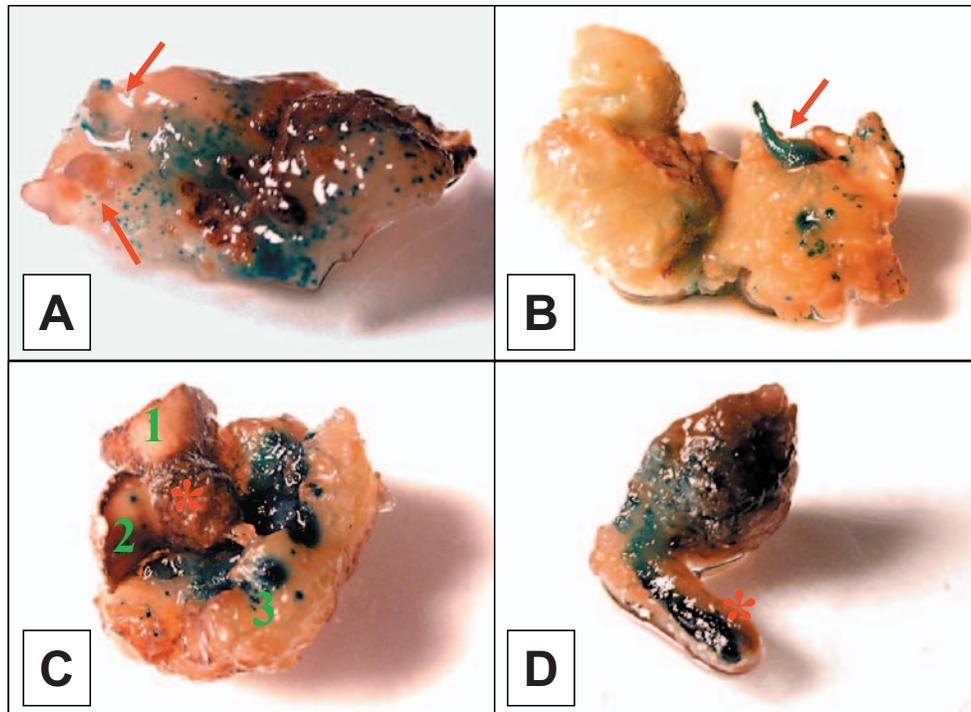
**Table 3.—Cumulative X-Gluc staining data for the 2003 experiment. Data are presented only for the subset of  $E_1$  and  $E_2$  embryos that were cultured on the same media type (selective vs. non-selective) as had been used for the  $E_0$  embryos.**

Treatment	Embryo Type <sup>1</sup>	N	GUS Positive		Staining Distribution (%) <sup>2</sup>			
			N	%	Small spots	Larger spots	Patches	Entire
Kanamycin Selection	$E_0$	44	44	100	89	18	25	0
	$E_1$ – Direct	39	37	87	82	12	3	16
	$E_1$ – Static	52	34	65	100	3	6	0
	$E_1$ – Growth	4	3	75	0	0	0	100
	$E_2$ – Direct	9	9		0	0	0	100
	$E_2$ – Growth	8	8		0	0	0	100
No Selection	$E_0$	38	28	74	61	21	29	0
	$E_1$ – Direct	27	8	26	43	14	0	43
	$E_1$ – Static	9	3	33	33	0	33	33
	$E_1$ – Growth	32	0	0	-	-	-	-
	$E_2$ – Direct	6	0	0	-	-	-	-
	$E_2$ – Growth	0	-	-	-	-	-	-

<sup>1</sup>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.

<sup>2</sup>Four basic staining patterns were recognized for the subset of embryos that tested GUS-positive: 1) chimeric staining consisting of smaller blue spots (< 0.5 mm diameter), 2) chimeric staining consisting of larger spots (> 0.5 mm diameter), 3) chimeric staining consisting of discrete patches or sectors of blue (at least 2-3 mm in size), and 4) continuous blue staining (wholly transgenic embryos). The percentage of embryos that were scored positive for each staining pattern is indicated. Note: Since some of the chimeric embryos scored positive for multiple categories, the percentages frequently add up to more than 100%.

<sup>4</sup>All of the  $E_2$  embryos evaluated were derived from the same two transformation events.

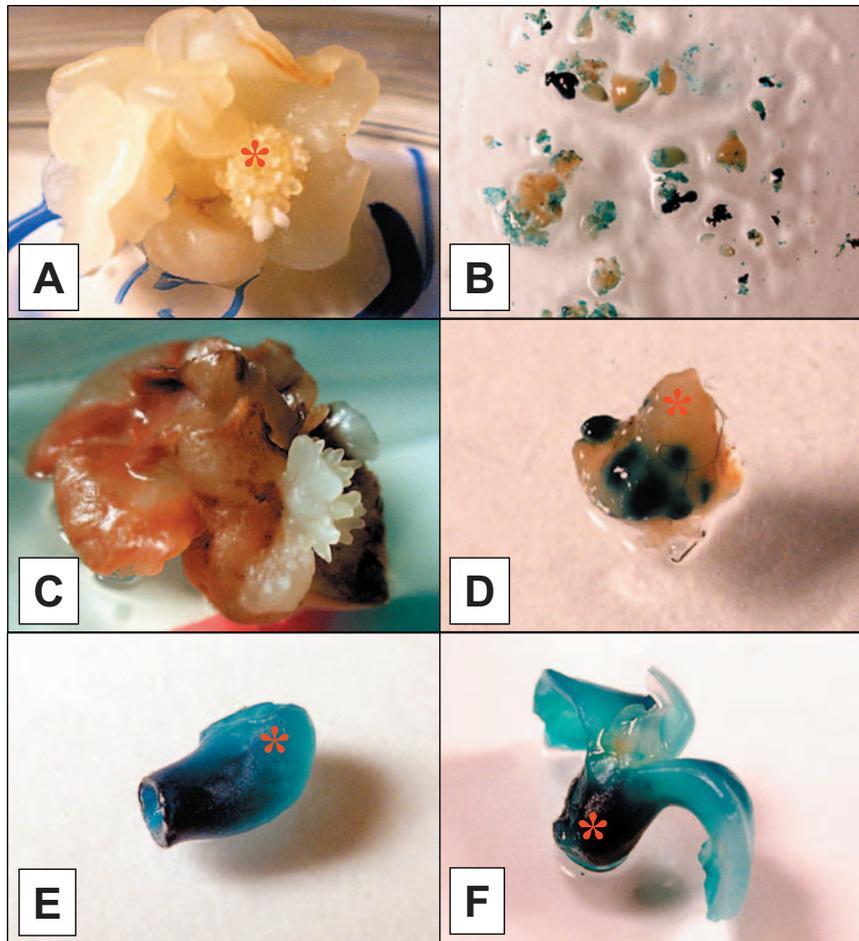


**Figure 3.—T-DNA transfer and GUS expression patterns in the infected embryos ( $E_0$  generation). (A) An  $E_0$  explant stained at the end of the first regeneration culture period. Notice the variable size of the blue spots. The smallest spots, which were estimated to be 35-50 micrometers in diameter may have represented single transgenic cells. Two secondary embryos ( $E_1$ ) are visible on the left side of the explant (arrows). One of the embryos is entirely unstained, while the second (upper) contains a few blue spots and differs little in staining pattern from that of the  $E_0$  embryo. (B) An explant stained at the end of the second culture period. The number of blue spots is more limited here; however, a wholly transgenic  $E_1$  embryo (with a tubular morphology, see arrow) has developed from one of the spots of GUS-positive cells. (C) An  $E_0$  embryo with three distinct cotyledons as viewed from below. Note the larger diameter of the blue spots for the cotyledon, which has grown in size since co-cultivation, compared with the size of the blue spots on the smaller (ungrown) cotyledons (labeled as 1 and 2). (D) An  $E_0$  embryo with spots of GUS positive cells on its cotyledon and a longitudinal band of GUS-positive cells along its hypocotyl-root axis, suggesting that most of the axis elongation has occurred since gene transfer (co-cultivation). Note: Asterisks indicate the root axes of the embryos (when visible).**

The frequency of GUS-positive  $E_1$  embryos varied widely between experimental treatments. When non-selective media was used for regeneration, only 16% (11/68) of the  $E_1$  embryos tested positive for GUS expression (Table 3; cumulative data, combined across embryo types). In addition, the degree of  $E_1$  embryo growth and the frequency of staining appeared to be inversely related (i.e., nearly 30% of the embryos that were stained at an early cotyledonary stage, tested positive for GUS activity; however, none of the embryos assayed after one or more culture periods of growth exhibited definite staining (Table 3). When kanamycin was included in the regeneration medium, positive staining results were obtained for nearly all of the  $E_1$  embryos evaluated directly (87%, Table 3), irrespective of the specific kanamycin concentration used (data not shown). The percentage of GUS-positive embryos was lower for the subset of embryos that were stained after one or more months of culture (65-75%, Table 3). However,

since most of the embryos had failed to exhibit de novo growth, the lower staining frequencies may have been a consequence of embryo quiescence rather than a lack of gene transfer.

The effects of the presence or absence of selection agent on the frequency of transgenic embryo regeneration has also been evaluated in *J. regia*. For the study in question (McGranahan and others 1990), the EHA105/pBI 121 *Agrobacterium* strain was used, 100 mg/L kanamycin was used for selection, and the  $E_1$  embryos were stained directly. However, only small samples from the  $E_1$  embryos were stained and the differences in staining frequency were even more dramatic than those observed here (i.e., only 0.05% of the embryos regenerated on non-selective media testing positive for GUS activity, compared with GUS-positive frequencies 31% for those regenerated on kanamycin medium).



**Figure 4.—Secondary embryo regeneration and GUS staining phenotypes. (A)** A mass of globular to polar-shaped embryos of indirect origin from its root pole of an  $E_0$  embryo on non-selective medium. **(B)** The X-Gluc staining patterns that were observed for a similar group of secondary embryos harvested from an explant on selective medium (500 mg/L kanamycin). Most of the embryos appeared either wholly transgenic or wholly non-transgenic. **(C)** A row of secondary embryos of direct origin from an  $E_0$  embryo cultured on 200 mg/L kanamycin medium. **(D)** An  $E_1$  embryo of direct origin following X-Gluc staining. Notice the larger spots of blue, some of which appear to be associated with spots of localized tissue growth which may represent early secondary embryos. The asterisk indicates the position of the embryo's root pole (facing upwards). **(E)** An  $E_2$  embryo that appears wholly transgenic as evidenced by its extensive blue coloration. **(F)** A larger, well-differentiated cotyledonary embryo from an established transgenic line. Note: Asterisks indicate the root axes of the embryos.

Despite the higher frequencies of transgenic embryo regeneration observed in the current study, most of the  $E_1$  embryos that tested positive for GUS staining were chimeric (Fig. 4D), irrespective of the experimental treatment (Table 3). Although some of the  $E_1$  embryos may have been present prior to co-cultivation, in which case chimeric staining would have been expected, very few of the  $E_0$  embryos bore secondary embryos at the time of inoculation, as assessed by visual inspection (data not shown). For the subset of  $E_1$  embryos regenerated on kanamycin medium, only 6/39 (16%) of the embryos that were directly stained appeared fully transgenic (100% blue). However, three additional transformation

events that had produced fully transgenic  $E_1$  embryos, but had been overlooked at the point of data collection, were identified in association with the evaluation of the stained  $E_0$  embryos. The percentages of GUS-positive  $E_1$  embryos that stained wholly blue was greater for the no-selection treatment (33-43%, Table 3); however, since the sample sizes of GUS-positive embryos were so small ( $n = 3-8$ ), these percentages may not be representative of the population-level means.

For the direct selection treatment, the percentage of wholly transgenic  $E_1$  embryos was related to the mode of origin of the secondary embryos and

**Table 4.—Relationships between secondary embryo origin, frequency of transformation events yielding wholly transgenic E<sub>1</sub> embryos<sup>1</sup>, and the regeneration timecourse for the 2003 experiment direct selection treatment.**

Origin <sup>2</sup>	N <sup>3</sup>	% Wholly GUS Positive	Regeneration Time Course <sup>4</sup>		
			Culture Period <sup>1</sup>	Culture Period <sup>2</sup>	Culture Period <sup>3</sup>
Direct-cotyledon	92	7	47	90	100
Direct-root	5	40	20	80	100
Indirect	4	75	0	25	100

<sup>1</sup> When regeneration was indirect, the initial harvested embryos presumably consisted of mixtures of E<sub>1</sub> embryos and subsequent embryo generations (E<sub>2</sub>, E<sub>3</sub>, etc). Wholly transgenic E<sub>1</sub> embryos were defined as those staining 100% blue when tested with X-Gluc (see Table 3)

<sup>2</sup> The transformation events were distinguished based on whether the E<sub>1</sub> embryos were of direct or indirect origin, with those of direct origin being further distinguished based on whether the secondary embryos were derived from the cotyledons or root of the parent (E<sub>0</sub>) embryo.

<sup>3</sup> The number of transformation events. Each transformation event consisted of one or more E<sub>1</sub> embryos that were located together on the surface of an E<sub>0</sub> embryos (i.e., within 5 mm of one another)

<sup>4</sup> For each class of E<sub>1</sub> embryos, the percentage of the total number of transformation events that were apparent at the end of each regeneration culture period is indicated.

the timing of embryo formation (Table 4). For most of the transformation events evaluated, the initial secondary embryos produced were of direct cotyledonary origin. The rate of secondary embryo formation was rapid, with 90% of the transformation events being apparent by the end of the second culture period; however, fully transgenic E<sub>1</sub> embryos were noted for only 7% of the transformation events. In contrast, for the subset of transformation events that involved the production of secondary embryos of either root or indirect origin, the frequencies of wholly GUS-positive E<sub>1</sub> embryos were much higher (40-75%) and the rate of regeneration was slower (Table 4).

Similar relationships between the frequency of GUS positive E<sub>1</sub> embryos and the rate of embryo regeneration have been observed for *J. regia* somatic embryos, as assessed via the staining of samples from the cotyledons of the E<sub>1</sub> embryos (McGranahan and others 1990). For the study in question only 5% of the embryos harvested within 6 weeks of co-cultivation tested positive for GUS activity, compared with GUS-positive frequencies of 11-40% for the subset of embryos harvested within 7-13 weeks of co-cultivation, and 55-65% for the subset harvested 14-16 weeks after co-cultivation. In addition, staining assays involving the evaluation of later generation embryos derived from the initial E<sub>1</sub> regenerants, indicated most of the embryos that tested positive at the E<sub>1</sub> stage had been wholly transgenic.

The relationships between the rate of embryo regeneration and percentage wholly transgenic secondary embryos observed here and by McGranahan and others (1990) can be interpreted in terms of differences in the degree of embryo development at the time of gene transfer. In

particular, secondary embryos transformed at a multicellular stage of development, which would exhibit chimeric staining, would be expected to appear prior to the emergence of embryos transformed at the unicellular stage, which would stain wholly blue. However, the higher frequencies of fully GUS-positive embryos that were observed for the transformation events involving the production of E<sub>1</sub> embryos of root or indirect origin that were observed in this study cannot be fully accounted for by differences in the rate of embryo regeneration. In fact, the frequency of transformation events yielding fully transgenic E<sub>1</sub> embryos was significantly lower for the transformation events involving direct embryo formation from the cotyledons of the infected embryos, even when the data was compared only for the subset of transformation events, where regeneration was not apparent until the end of the third culture period (data not shown). For the transformation events involving indirect embryo proliferation, the regeneration of stable transgenic lines may have been facilitated by the precocious proliferation of chimeric E<sub>1</sub> embryos prior to the onset of a state of embryo quiescence (see below).

### E<sub>1</sub> Proliferation and Establishment of Transgenic Lines

For the 2003 experiment, none of the E<sub>1</sub> embryos that were regenerated on kanamycin-free media continued to grow or proliferate following transfer to kanamycin media for either of the concentrations tested. *De novo* growth and proliferation were observed for most of the embryos kept on non-selective medium (Table 5); however, none of the E<sub>2</sub> embryos produced tested positive for GUS activity (Table 3). The number of embryos evaluated

was limited (6-35 depending on the parameter); however, similar results were obtained for *J. regia* by Tang and others (2002) (i.e., when non-selective regeneration media was used, the frequency of GUS-positive E<sub>2</sub> embryos ranged from 0-2% depending on the antibiotic used for Agrobacterium elimination). In contrast, when kanamycin (100 mg/L) was used for selection, 14-22% of the embryos tested GUS positive.

The recalcitrance of the E<sub>1</sub> embryos regenerated on kanamycin medium for continued growth and proliferation was unexpected. Irrespective of the type of media used (selective or non-selective), less than 10% of the subcultured E<sub>1</sub> embryos exhibited *de novo* growth (Table 5). *De novo* growth was observed for three of the embryos transferred to non-selective medium (3/34) and two of the three produced additional generations of secondary embryos; however, none of the E<sub>2</sub>/E<sub>3</sub> embryos tested positive for GUS activity (0/14) and none

were kanamycin tolerant (0/12). In contrast, for five of the seven E<sub>1</sub> embryos that were maintained on selective medium and exhibited *de novo* growth, stable transgenic lines (representing two distinct transformation events) were obtained. Although only two of the five E<sub>1</sub> embryos that yielded transgenic lines were evaluated by X-Gluc assay, both stained fully blue. In contrast, none of the E<sub>1</sub> embryos from the selective media plates that had failed to grow or proliferate following harvest stained 100% blue (Table 3).

For the combined data sets (2002, 2003) three distinct transgenic lines, representing separate embryo genotypes, were obtained. However, all three of the lines were derived from E<sub>0</sub> embryos that had been cultured on regeneration media with 200-250 mg/L kanamycin, suggesting that some or all of the kanamycin concentrations used in this study (200-500 mg/L) may have been supraoptimal for line establishment. In a

**Table 5.—Growth and proliferation responses of E<sub>1</sub> embryos from the 2003 experiment. The data is sorted by experimental treatment and media type, but is combined across embryo lines and kanamycin (Kan) concentrations.**

Treatment	Media	N	% Growth <sup>1</sup>	% Prolif <sup>2</sup>	% Producing Stable Lines <sup>3</sup>
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

<sup>1</sup> Percentage with definite cotyledon enlargement (to at least 5 mm in maximal dimension).

<sup>2</sup> Percentage producing secondary embryos, typically observed after 2-3 culture periods.

<sup>3</sup> Percentage giving rise to stable kanamycin-resistant lines.

**Table 6.—Kanamycin (Kan) sensitivity of non-transgenic walnut somatic embryos. The data presented is pooled from two experiments. For each experiment embryos from 4 to 8 lines were evaluated, with two to four embryos from each line being evaluated per media type. The cumulative data (collected after 5-6 wks of culture and pooled across embryo sources) are presented. Proliferation data are presented only for the subset of embryo lines that had produced secondary embryos on control medium (without kanamycin).**

Media (mg/L Kan)	Small Cotyledonary <sup>1</sup>			Large Cotyledonary <sup>2</sup>	
	N <sup>3</sup>	% Grown <sup>4</sup>	% Prolif <sup>5</sup>	N	% Prolif <sup>5</sup>
0	29 (18)	79	75	16	44
25	21 (14)	81	64	16	50
50	28 (19)	75	16	16	31
100	29 (18)	72	0	16	0
250	29 (18)	21	17 <sup>A</sup>	24	12 <sup>A</sup>
500	29 (18)	3	0	24	0
1000	29 (18)	0	0	24	0

<sup>1</sup> The embryos were 2-5 mm in total length with cotyledons 1-3 mm long.

<sup>2</sup> The embryos were 10-20 mm in dimension with cotyledons 7-15 mm long.

<sup>3</sup> The sample size for the % proliferation data is indicated in parentheses.

<sup>4</sup> % embryos with cotyledon enlargement as visually assessed.

<sup>5</sup> % embryos producing secondary embryos.

<sup>A</sup> For two-thirds of the embryos that exhibited proliferation on 250 mg/L kanamycin medium, the secondary embryos produced were of indirect, rather than direct, origin.

series of preliminary kanamycin dose-response experiments, embryo proliferation was only erratically observed at kanamycin concentrations of 100 mg/L or greater (Table 6), and for several embryo lines, the secondary embryos produced in the presence of kanamycin exhibited retarded cotyledon differentiation and would not have been expected to produce additional generations of embryos of direct origin. Similarly, sub-lethal doses of kanamycin have been observed to antagonize shoot regeneration in several plant species (Yepes and Aldwinkle 1994, Tosca and others 1996, Peros and others 1998, Humara and Ordas 1999, Kapaun and Cheng 1999). In addition, in some studies, kanamycin has been found to interfere with plant regeneration even following NPT II gene incorporation (Mullins and others 1990, Robertson and others 1992, Ellis and others 1993, Aldemita and Hodges 1996, Azhakanandam and others 2000).

For *J. regia*, 100 mg/L kanamycin have routinely been used for the selection of transgenic embryos (McGranahan and others 1990; Dandekar and others 1989, 1998; Escobar and others 2000; Tang and others 2000); however, this medium has been shown to be ineffective for the exclusion of non-transgenic secondary embryos in multiple labs (McGranahan and others 1990, Tang and others 2000). In addition, for certain *J. regia* x *J. nigra* lines the regeneration of non-transformed escapes has been observed even at kanamycin concentrations of up to 500 mg/L. However, in contrast with the results obtained in this study, when media with 100 mg/L kanamycin were used, the continued growth and proliferation of chimeric E<sub>1</sub> regenerants was possible (McGranahan and others 1990, Tang and others 2000).

The delayed kanamycin sensitivity that was exhibited by the chimeric secondary embryos regenerated in this study suggests that the degree to which populations of transgenic black walnut cells are able to protect an embryo from the inhibitory effects of kanamycin exposure may be related to the size of the embryo and/or its level of tissue differentiation. During the initial stages of somatic embryogenesis, the transgenic sectors of the chimeric E<sub>1</sub> regenerants were apparently able to cross-protect adjacent non-transgenic cells. However, with continued (symmetric) embryo growth, the ability of the transgenic cells to provide immunity for the entire E<sub>1</sub> embryo decreased, presumably as a result of the increased spacing between the transgenic and non-transgenic cell groups and/or changes in NPT II diffusion/transport characteristics, to such a degree that the competence of the embryos for continued growth and proliferation was ameliorated even prior to the point of embryo harvest.

## CONCLUSIONS AND FUTURE WORK

Despite the high rates of gene transfer and integration that were documented across multiple genotypes, robust protocols for the selection of transgenic *J. nigra* embryos and subsequent establishment of transgenic lines remain to be developed. When kanamycin (200-500 mg/L) was used for selection, nearly all of the initial regenerants were transgenic; however, most were chimeric and appeared incapable of continued growth and line establishment, at least via a direct proliferation pathway. Future investigations in our lab will focus on a more systematic evaluation of the kanamycin sensitivity of black walnut somatic embryos (both in the presence and absence of a heterologous NPT II gene), with the primary objective of identifying kanamycin concentrations that could be used to block non-transgenic regeneration without inhibiting the subsequent growth and proliferation of chimeric secondary embryos, which accounted for the majority of the regenerants in this and other studies. Other research possibilities include an evaluation of the effects of E<sub>0</sub> embryo size on the transformation response. The use of smaller (younger) embryos for infection would presumably decrease the likelihood of secondary embryo formation prior to gene transfer, resulting in reductions in the frequency of chimeric embryo regeneration. However, for high transformation rates to be achieved, the initial transgenic cells would need to be able to divide relatively freely, so as to produce populations of transgenic cells (i.e., entire blue sectors or patches) from which embryos were subsequently regenerated.

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