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RESPONSES OF DIFFERENT GENOTYPES OF ROBINIA PSEUDOACACIA L. TO MICROPROPAGATION AND AGROBACTERIUM-MEDIATED TRANSFORMATION

By

John Mark Davis

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ABSTRACT

RESPONSES OF DIFFERENT GENOTYPES OF ROBINIA PSEUDOACACIA L. TO MICROPROPAGATION AND AGROBACTERIUM-MEDIATED TRANSFORMATION

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The objectives of these experiments were to assess the feasibility of using *in vitro* techniques to micropropagate mature *Robinia pseudoacacia* (black locust) trees, and to introduce foreign DNA into the genome of this species by the use of *Agrobacterium*. These tools would be useful for increasing the efficiency of an applied breeding program, and also for basic research efforts which require gene transfer.

To conduct the micropropagation study, stem segments were collected from five mature trees during winter dormancy. Buds were excised from the stems and cultured on MS salts and vitamins medium containing various levels of 6-benzylaminopurine. Tree-specific responses to bud culture were observed; only two of the five trees produced proliferating shoot cultures. Clone-specific effects on rooting were also observed in the two trees that were successfully propagated. These results indicated that some mature black locust trees can be efficiently micropropagated, while others tend to be recalcitrant to *in vitro* growth.

Agrobacterium-mediated gene transfer of T-DNA to the black locust genome was demonstrated by inciting tumor formation on seedling explants, and examining the DNA from these tumor lines by Southern analysis. T-DNA inserts from three different opine-negative, phytohormone-independent tissue lines were found to be truncated on the right end. The left-hand junction fragments appeared normal in the tumors that were examined, which makes it seem unlikely that there is a fundamental barrier to T-DNA transfer, integration, or expression in black locust. When explants were inoculated with modified *Agrobacterium* strains, which contained selectable marker loci within the T-region, kanamycin resistant calli were obtained. Southern analysis indicated that the loci were integrated into the black locust genome. However, maintenance of vigorous callus growth, and regeneration of transgenic callus into plantlets are still barriers to the integration of this technique into basic and applied research programs.

This dissertation is dedicated
to my parents,
Melvin H. and Frances L. Davis

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Bacterial strains used in these experiments were kindly provided by Dr. Mike Thomashow (*A. tumefaciens* strains and T-region clones), Dr. Frank White (*A. rhizogenes* strains), and Drs. Gene An, Georgia Helmer, and Barb Baker (all of whom provided strains with modified T-regions).

Finally, I express my love and gratitude to my wife, Kathy, who has been so understanding and supportive during the course of my graduate studies.

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INTRODUCTION

Robinia pseudoacacia L. (black locust) has several characteristics which make it an especially promising candidate for tree improvement efforts. *Robinia* is the second most widely planted hardwood genus in the world (Keresztezi, 1980), so genetic gains in a breeding program would potentially have widespread impact. In addition, enough genetic variability exists for most important traits in black locust to warrant improvement efforts (Kennedy, 1983; Mebrahtu and Hanover, 1989).

Utilization. Black locust wood was widely used in the early part of this century for fenceposts, insulator pins, wagon wheel hubs, and treenails for ship building (Cuno, 1930). These uses required wood that was sturdy, resistant to decay, and demonstrated minimum shrinkage in response to moisture changes. Black locust wood fulfilled those requirements, and also provided an excellent source of fuelwood. It nodulates in association with *Rhizobium* (Allen and Allen, 1981), and can therefore thrive on poor sites that would exclude many other tree species. Black locust is a desirable species in short rotation, intensive culture systems for use as an energy source (Miller *et al.*, 1987), as a high quality animal feed (Baertsche *et al.*, 1986), or as a multipurpose tree species (Barrett *et al.*, 1988). In addition, trees often begin to flower after four years of vegetative growth. This characteristic of early, often

prolific, flowering has formed the basis for commercial honey production in Hungary (Keresztezi, 1983, 1988), and also reduces the time required (relative to most other tree species) to complete a generation of tree improvement work.

Historically, the major limitation to the growth of black locust in the U.S. has been the susceptibility of the species to the locust borer (*Megacyllene robiniae* Forster) (Hoffard and Anderson, 1982). The larvae of this insect pest tunnel into the bole of black locust trees, making the wood worthless for dimension lumber. The damage caused by the insect also makes trees susceptible to windthrow, provides entry points for secondary pathogens such as the heartrot fungus *Fomes rimosus*, and usually ruins stem form (Hoffard and Anderson, 1982). Some black locust clones appeared to be genetically resistant to the locust borer (Hopp, 1941). However, according to Huang *et al.* (1975), the design of subsequent field tests of these clones did not allow this hypothesis to be tested. On the basis of results obtained through the use of biochemical systematics, they suggested the "shipmast locust" would be better described as an ecological variant, not a true variety.

Need for in vitro methods. *Robinia pseudoacacia* has several characteristics which make it a promising species for use in tissue culture systems. It shows rapid growth rates during the juvenile phase, often exceeding 2 m/yr (Barrett *et al.*, 1988). This should minimize the time interval necessary to obtain plantlets which are ready for transfer to the greenhouse and field. The growth habit is indeterminate, and it vegetatively regenerates vigorously from root sprouts and by coppicing (NAS, 1983; Keresztezi, 1988), and many species which exhibit these characteristics also show desirable *in vitro* responses (Mott, 1981). In fact, shoots were obtained from shoot tip callus (Brown and Sommer, 1982), nodal segments (Chalupa, 1983), and leaf explants (Davis and Keathley, 1985) of young black locust trees prior to the initiation of the studies reported in this dissertation.

The incorporation of *in vitro* micropropagation into forest tree improvement programs has been summarized by several authors (Durzan and Campbell, 1974; Karnosky, 1981; Mascarenhas *et al.*, 1987). If the goal is the production of genetically elite planting stock, then micropropagation of superior individuals would allow the mass production of those genotypes. Alternatively, if the goal is to increase the amount of genetically improved seed which can be obtained in a given generation of tree breeding, then micropropagation would allow the replication of individuals with high breeding values for use in clonal seed orchards. These strategies allow the breeder to capture the maximum amount of genetic gain per generation, helping to minimize the negative impact of lengthy generation intervals inherent to tree populations.

In both cases, the ability to use mature trees as a source of tissue is desirable. In the first case, the tree of interest would normally have reached maturity by the time its genetic superiority had been assessed. This is because most traits of interest to the tree breeder, such as stem diameter at harvest age, or resistance to wood-boring insects, can only be assessed after the tree has matured. In the second case, the tree of interest must flower in order for its breeding value to be assessed by progeny tests.

In order for these concepts to be applied to the black locust breeding program, methods for micropropagation of mature black locust trees must be developed. This is an important objective, since propagation using root or stem cuttings with mature trees yielded less than 4 propagules, on average, from each root or stem segment (Prentice, 1987). Although recent experiments indicate that propagation using root segments can be used to clone most black locust genotypes (Dr. Tesfai Mebrahtu, personal communication), such procedures can cause significant damage to the donor tree. It is also not yet clear whether clone-specific effects will be observed in the rooting of the sprouts. *In vitro* propagation, alone or in combination with conventional propagation, may allow the efficiency of the cloning process to be increased. This strategy was explored in the experiments that are reported in the first chapter of this dissertation.

While micropropagation can be used to exploit genetic variability which exists in the breeding population, it can not be used to predictably change the genotype of any particular individual. For example, micropropagation alone can not be used to address the lack of borer resistance in black locust, unless a resistant genotype is discovered. However, *Agrobacterium*-based vectors have been utilized to introduce insect resistance into crop plants such as tobacco, (Vaeck *et al.*, 1987), which suggests that this may be a useful strategy for application to other plant species, including black locust. In addition, these vectors have demonstrated potential as powerful tools for basic research on the regulation of gene expression in plants (Kuhlemeyer *et al.*, 1987; Klee *et al.*, 1987). The introduction of foreign genes into the somatic tissues of black locust *in vitro* was explored in the experiments which are reported in the second and third chapters of this dissertation.

Objective. The overall objective of the studies reported in this dissertation was to examine the feasibility of applying the techniques of mature tree micropropagation and *Agrobacterium*-mediated gene transfer to a tree improvement program for *R. pseudoacacia*. The first chapter describes the development of a bud culture technique for mature black locust trees, and the explant source-specific responses to this culture system are discussed. In the second chapter, the stable transfer of foreign DNA from *Agrobacterium tumefaciens* to a woody legume is demonstrated for the first time. The third chapter describes the use of modified *Agrobacterium* strains to transfer loci encoding resistance to aminoglycoside antibiotics into black locust cells. Conclusions and recommendations, based on the results of these studies, are presented at the end of this dissertation.

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CHAPTER 1

Differential responses to *in vitro* culture in mature *Robinia pseudoacacia* L. (black locust)

ABSTRACT

Micropropagation of five selected 20- to 30-year old black locust (*Robinia pseudoacacia*) trees by *in vitro* culture of surface sterilized stem segments was attempted. These efforts resulted in the successful initiation of shoot cultures from one tree. Out of a total of 312 explants, only six aseptic shoots were obtained from this tree. The remainder of the explants in the experiment from the other four trees (1,080) were lost to contamination.

To circumvent this explant contamination problem, buds were excised from the stems of the same five trees just prior to natural bud flush in the spring, and were placed on MS basal medium with various levels of 6-benzylaminopurine (BAP). Tree-specific differences were observed with respect to the average number of contaminated bud explants in these experiments, but contamination was not a limiting factor to the successful initiation of cultures from any tree. In all media tested, bud explants from two of the trees produced shoots which proliferated and could be subcultured. Explants from three other trees became vitrified or produced callus on the tested media; no proliferating shoot cultures which could be subcultured were obtained from these trees.

After 5 months of culture on medium containing 0.32 uM BAP, 134 shoots (2 to 3 cm in length) from the two trees which were successfully propagated, were placed on 0.1 strength MS medium containing 1 uM naphthaleneacetic acid (NAA), 1 uM indolebutyric acid (IBA), or 0.5 uM NAA + 0.5 uM IBA. The tree that responded best to the bud culture and shoot proliferation media also had the highest efficiency of rooting. The differences in percent rooting between the two explant sources were much larger than the differences observed due to the culture medium treatments. A higher proportion of shoots from both explant sources produced roots on medium that contained NAA, but the roots detached from the shoot upon removal of the plantlet from the agar medium.

These results suggest that forest tree breeding programs which plan to use micropropagation should consider using *in vitro* performance as part of the selection scheme.

INTRODUCTION

Black locust (*Robinia pseudoacacia* L.) is a papilionaceous legume which is native to the southeastern United States, but has a naturalized range that extends worldwide (Harlow et al., 1979). This species has desirable characteristics which include rapid vegetative growth and early, often prolific, flowering. This makes black locust a good forage for honeybees (Keresztezi, 1984). It also has high density wood that is excellent for fuel (NAS, 1983) and is highly resistant to decay (Keresztezi, 1980). Black locust is also well known for its utility in reclaiming poor soils by virtue of its association with nitrogen-fixing *Rhizobium* (Allen and Allen, 1972).

Some reports have indicated that juvenile black locust trees can be successfully propagated *in vitro* (Brown and Sommer, 1982; Chalupa, 1983, 1987; Davis and Keathley, 1985). Such reports, however, do not provide evidence that this species is amenable to mature tree micropropagation systems which are designed to clone elite forest tree genotypes (the goals of these systems have been discussed in Durzan and Campbell, 1974; Karnosky, 1981; Mascarenhas *et al.*, 1988). There are several barriers to the use of mature trees as explant sources (reviewed in Bonga, 1982; Zimmerman, 1984), including contamination of explants by microorganisms, secretion of growth-inhibiting metabolites by the explant in culture, and lack of vigorous growth on media which support growth of juvenile tissues.

Similar problems due to the lack of vigorous tissue growth *in vitro* have been addressed in herbaceous legumes by the use of specific lines, cultivars, or varieties which grow and differentiate readily. Highly morphogenic lines of diploid alfalfa (Bingham *et al.*, 1975; Reisch and Bingham, 1980) and red clover (Keyes *et al.*, 1980) have been developed for use in tissue culture, physiological, and molecular biological research. A similar approach could prove useful for obtaining desirable performers in forest tree legumes such as black locust. The overall goal of the experiments reported in this chapter was to determine if mature black locust trees which grow vigorously in culture could be identified.

MATERIALS AND METHODS

The initial barrier to successful culture was explant contamination. After culture contamination was reduced to an acceptable level, the systematic study of *in vitro* shoot cultures from mature explants of black locust could be initiated. To address the contamination problem, two sets of experiments were performed. In the first experiment, stems were cut into segments possessing a single bud. After a surface sterilization treatment, these segments were inserted vertically in agar-solidified medium to stimulate bud elongation. This experiment is referred to as the "stem culture" experiment in this paper. In the second set of experiments, buds were excised from the stems, and only the

isolated buds were used as explants on medium containing various levels of BAP ("bud culture" experiments).

Five black locust trees were selected from four different locations (two trees, #2 and #3, were from the same uneven-aged stand) in southern Michigan, to minimize the possibility that the chosen trees were closely related. Age was determined for each tree by counting annual rings on a core sample taken at breast height with an increment borer.

In all experiments, branch tips were removed from each tree, placed in double-distilled water, and the stems were transported to the lab. To reduce stem surface contaminants, stems were cut into 20- to 30-cm segments, placed in a 500 ml graduated cylinder, and immersed in a sterilizing solution of 2.5% NaOCl with 10 drops/l Tween-20 for 20 minutes, followed by three separate one-volume rinses with sterile double-distilled water. The stem segments were allowed to stand in a fourth rinse for 5 min, after which the water was decanted.

Stem culture. Cuttings were placed in sterile, moistened paper towelling, irrigated with 150 ml of carbenicillin solution (500 ug/ml), sealed in aluminum foil, and incubated at 25^{o}C for three days. The NaOCl sterilization treatment described above was then repeated, and stems were cut into segments with one bud each. The bases of the segments were then inserted into 5 ml of agar-solidified (0.8% w/v) MS (Murashige and Skoog, 1962) medium

containing 1 uM BAP in each 18x150 mm culture tube. After four weeks, the explants were examined for contamination (Table 1).

Bud culture. Stem segments were removed singly from the cylinder and placed on sterile filter paper for excision of lateral buds. The dormant buds of black locust lack true bud scales, are 1-2 mm in length, and lie sunken beneath three apparent "plates" of the leaf scar (Harlow et al., 1979). The basal attachment of each plate was cut, and the plates were removed to expose the buds. The buds were then excised (Figure 1) and placed vertically in 100x15 mm Petri dishes, each of which contained 25 ml of MS basal medium with 1.2% agar (w/v) and various levels of BAP. Two bud culture experiments were performed; in the second experiment, BAP levels were ten-fold higher than in the first experiment (Table 3). The general range of BAP concentrations was selected on the basis of preliminary studies and other published reports (Chalupa, 1983; Davis and Keathley, 1985). Four buds were cultured in each dish, and five dishes were used for each phytohormone treatment (20 buds/tree/ phytohormone level).

Three days after excision from the tree, and every 4 to 7 days thereafter, buds were transferred individually to 10 ml of fresh medium in each 25x150 mm culture tube. Transfers were relatively frequent, because results from initial experiments using stem cuttings indicated that mature black locust tissues secrete metabolites which inhibit growth into the culture medium. Necrotic tissue, if present, was trimmed from the base of each explant at the time of transfer. Twenty-three days after excision from the tree, data were collected on the number of shoots per aseptically cultured bud, and the length of the longest shoot from each of six randomly chosen explants in each treatment. The number of cultures which had been lost to contamination was also counted at that time.

Individual shoots were then excised from the primary bud explants, and transferred to fresh MS medium containing 0.32 uM BAP for elongation. This BAP level was selected based on data from Davis and Han (unpublished). The shoots were maintained on this medium for a total of 24 days, with one transfer to fresh medium after 12 days. Data were then collected on the number of non-vitrified shoots produced (Table 4).

Only shoots from trees #1 and #4 produced shoot cultures which proliferated enough to perform a rooting study. Cultures were maintained on MS medium containing 0.32 uM BAP for 5 months (4 week subculture interval). Shoots which had attained a length of 2 to 3 cm were individually transferred to culture jars containing 50 ml of 1/10 strength MS basal medium with no sucrose, 1.0% (w/v) agar, and auxin. Auxin treatments were either 1.0 uM IBA, 1.0 uM NAA, or 0.5 uM IBA + 0.5 uM NAA. A total of 22 shoots/auxin treatment/explant source were cultured. Each week, for a total of four weeks, the number of shoots which produced roots was counted. Root diameters were measured using a micrometer and a dissecting microscope. For all experiments, cultures were placed in a growth chamber maintained at 26-27°C, with an 18 hr light/6 hr dark lighting regime (75-100 umol/s/m^2 PAR).

RESULTS

Stem culture. Preliminary data suggested that a second treatment in the NaOCl solution after 2-3 days of incubation in a moist environment reduced contamination. This was presumably due to increased susceptibility of surface microorganisms to the sterilant after the germination of resting spores. Ten out of 1,392 (0.7%) of the stem segments which were cultured in this experiment showed no visible signs of contamination (Table 1). The buds from four of these segments did not elongate, probably due to damage sustained during the sterilization treatments. A total of six shoot cultures were initiated as a result of these efforts. However, since all were obtained from tree

Table 1. Number of explants that were aseptic after four weeks of culture.

Tree	Age	No. of explants	No. of aseptic stem segments	No. of aseptic shoots
1	21	312	8	6
2	24	279	2	0
3	30	279	0	-
4	20	280	0	-
5	27	242	0	-

#1, this method of surface sterilization did not allow systematic study of all five trees *in vitro*.

Bud culture. The contamination results obtained using bud culture (Table 2) showed that the overall percentage of the explants which had visible fungal or bacterial contamination was 19.7%, but this percentage varied among the trees which were tested (results from the first and second bud culture experiments were similar). Values ranged from 1.7% (tree #3) to 38.3% (tree #4), and trees #1, #3, and #5 had significantly lower levels of contamination than trees #2 and #4 (Duncan's NMR test, alpha = .05).

The average number of shoot meristems per cultured bud, and the average length of the longest shoot per bud explant for the first bud culture experiment, are shown in Table 3 (Experiment 1 section). More than one center of shoot proliferation was present on many of these bud explants,

Table 2. The average number and total percentage of contaminated explants for five black locust tree selections.

Tree	BAP level (uM)			Average Number of Contaminated Explants	Total (%)[1]
	0.32	1.0	3.2		
1	1[2]	3	0	1.3 a	6.7
2	7	5	5	5.7 c	28.3
3	0	1	0	0.3 a	1.7
4	9	9	5	7.7 c	38.3
5	3	5	1	3.0 ab	15.0

[1] Averages which have different letters are significantly different at alpha = 0.05, using Duncan's New Multiple Range test.

[2] Twenty observations per datum.

since black locust buds are often superposed (Harlow et al., 1979; Figure 2). Buds from tree #1 produced the longest shoots, trees #3 and #5 the shortest, and the other trees were intermediate. The same hierarchy was apparent when values for the average number of shoots per explant were compared.

The data in Table 3 (Experiment 1), indicated that the average length of the longest shoot increased with increasing concentrations of BAP for all five trees. This is consistent with the AOV of the shoot length data, which showed that the cytokinin level (and the explant source, as well) had a highly significant effect on shoot elongation (F-test, alpha = 0.01). In addition, the average number of shoots per bud from trees #3 and #5 was less than 1.0. Taken together, these data suggested that higher BAP levels should be tested in a second experiment.

Branch tips were again harvested, and the buds were excised as in the first experiment. The 0.32 uM BAP treatment was included in the second experiment to test the repeatability of the results obtained in the first. Both the average number of shoots produced, and the average length of the longest shoot per bud, were compared across the two 0.32 uM BAP treatments for each tree (Table 3, middle columns). None of the averages for shoot number or shoot length differed significantly when tested pair-wise across the two experiments (t-test, alpha = 0.05). Shoot number and shoot length did not continue to increase significantly in the second experiment. This is reflected in the AOV of these data, which indicated that only differences due to the explant source were significant in explaining differences in shoot number or length (F-test, alpha = 0.01). As in the first experiment, buds from tree #1 produced the longest (and most) shoots, trees #3 and #5 the shortest (and fewest), and the other two trees had shoots of intermediate length.

The treatment that produced the most shoots longer than 1.0 cm for each tree is listed in Table 4. The response of buds from trees #1 and #4 was uniform across BAP levels, as indicated by the relatively low percentage of the total shoots which were produced in the best treatment. In contrast, almost all of the shoots which were produced by buds from trees #2 and #5 came from the 3.2 uM BAP treatment.

In an initial rooting experiment, only shoots from trees #1 (82%) and #4 (21%) produced roots after 3 weeks on rooting medium. No analysis of these preliminary data was attempted due to unequal sample sizes and the confounding of possible carry-over effects (Keathley and Scholl, 1982) from the different initial bud culture treatments. Shoot cultures from trees #1 (Figure 3) and #4 were maintained by subculturing (4 week interval) on MS containing 0.32 uM BAP for 5 months prior to a large scale rooting study. No decline in the viability of these cultures was detected.

Table 3. Average number and length of shoots produced by five black locust tree selections.

		BAP level (uM)					
		Experiment 1			Experiment 2		
	Tree	0.032	0.1	0.32	0.32	1.0	3.2
Average Shoots per Explant[1]	1	0.8	1.8	2.6	1.7	2.4	2.1
	2	0.7	1.5	1.5	1.2	1.3	1.7
	3	0.2	0.6	0.5	0.7	0.6	0.5
	4	0.8	1.6	1.7	1.7	1.8	1.6
	5	0.0	0.4	0.6	0.6	0.9	0.9
Average Shoot Length[2] (mm)	1	2.2	4.0	6.5	6.2	4.3	4.0
	2	0.3	2.3	2.3	2.3	1.5	2.5
	3	0.7	1.2	1.2	1.3	0.5	0.8
	4	1.0	2.5	3.0	3.3	3.7	3.5
	5	0.0	0.8	0.8	1.0	0.8	1.2

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[1] Averages were calculated from the number of visible shoot meristems 23 days after culture initiation. Only aseptic cultures were scored.

[2] Averages were calculated from the length of the longest shoot in each of six randomly chosen culture tubes.

Table 4. The number of shoots produced by explants for each black locust tree in the best treatment (data from Experiment 2). Data were collected 24 days after excision of shoots from the primary explant.

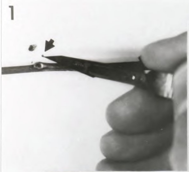
Tree	Best Treatment BAP (uM)	Shoots <1.0 cm	Shoots >1.0 cm	Total number of shoots[1]
1	1.0	18	13	90
2	3.2	3	1	6
3	1.0	1	0	1
4	1.0	8	7	53
5	3.2	9	3	17

[1] A total of sixty buds were cultured per tree in Experiment 2 (twenty buds per treatment).

Cultures from trees #2 (Figure 4), #3, and #5 remained recalcitrant, and eventually declined in vigor to where they could not be maintained. All the propagules from tree #1 which were transferred to soil appeared phenotypically normal 5 months after culture initiation (3 months after transfer to the greenhouse)(Figure 5).

The experiment designed to test the rooting responses of the shoots from trees #1 and #4 is summarized in Figure 6. Tree-specific differences were observed between trees #1 and #4 with respect to the number of shoot explants which produced roots over the course of four weeks in the culture medium. An average of 91% of the shoots from tree #1 produced roots; an average of 36% of the shoots from tree #4 produced roots.

The presence of IBA alone in the culture medium resulted in slower formation of roots, in comparison to



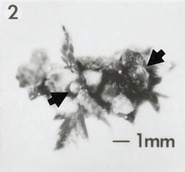


Fig. 1. Black locust bud (arrow) after excision from a stem.

Fig. 2. Bud explant from tree #1 after 12 days on medium containing 0.1 uM BAP. Arrows point to shoot apices.

Fig. 3. Shoot culture from tree #1 (tube = 25 mm diam).

Fig. 4. Non-proliferating shoot culture from tree #2 (tube = 25 mm diam).

Fig. 5. Plantlet from tree #1, 5 months after culture initiation.

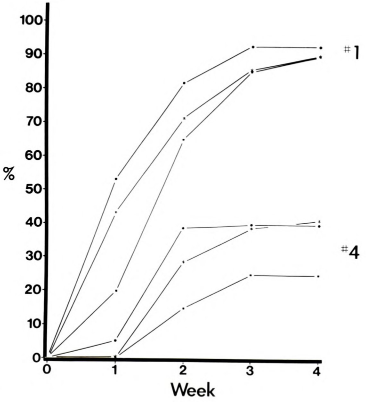


Figure 6. Cumulative percentage of shoots from trees #1 and #4 which produced at least one root on 1/10 strength MS medium containing either (▪) 1 uM IBA, (•) 1 uM NAA, or (*) 0.5 uM NAA + 0.5 uM IBA. Twenty-two shoots 2-3 cm in length were cultured per treatment.

medium containing NAA (Figure 6). On average, roots formed in the presence of IBA alone were more narrow in diameter (0.66 mm) than those induced in the presence of NAA (1.44 mm; Table 5). AOV showed that explant source, medium type, and the explant x medium interaction were all significant in explaining differences in root diameter (F-test; alpha = .01). When plantlets were removed from the culture jars after 6 weeks of culture, many of the roots (55%) that had been induced in medium containing NAA, detached from the shoot and remained in the agar medium. In contrast, roots induced in the presence of IBA remained attached to the shoot (Table 5).

DISCUSSION

Explant contamination. Explant contamination can be a significant, although often overlooked, barrier to the successful initiation of cultures from mature trees. Similar results obtained with spruce cuttings (Keathley, 1984) led to the alternative strategy of bud culture.

Contamination was not a random event with respect to each bud which was cultured, but was partly due to tree-specific factors. Possible factors are stem diameter, leaf scar size, the width of the gaps which are present between the plates of the leaf scar, and pubescence of the plate margins. Stem diameter and leaf scar size dictate the angle at which the incisions to remove the plates are made, and increased gap width and plate pubescence may influence the

Table 5. The average diameter (mm) of roots that originated from the bases of shoot explants derived from trees #1 and #4, and the frequency of root detachment upon removal of the shoots from agar-solidified culture medium containing 1.0 uM IBA, 1 uM NAA, or 0.5 uM IBA + 0.5 uM NAA. Data were collected from 5 randomly chosen plantlets at the end of the sixth week of the rooting experiment.

		Auxin Treatment		
Tree		IBA	IBA+NAA	NAA
1	Avg root diameter:	0.60[1]	1.22	0.94
	Detachment:	0/5	3/5	1/5
4	Avg root diameter:	0.72	1.50	2.09
	Detachment:	0/5	3/5	4/5

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[1] Root diameter measurements were made 5 mm from the point of root-shoot attachment on the largest root that had emerged.

effectiveness of surface sterilization of the leaf scar by trapping air bubbles.

The ease with which aseptic cultures were initiated from each tree, using the bud excision technique, was not a good indicator of overall performance *in vitro*. Contamination was low, and more shoots were produced by the buds from tree #1 than any other tree in both bud culture experiments. Tree #4 was also readily propagated, but in contrast to tree #1, had the highest frequency of explant contamination in the experiment. Explant contamination was almost absent in the buds cultured from tree #3, but the shoots which were produced on these explants did not continue to grow. Although contamination affected the efficiency with which cultures could be initiated, it was not the limiting factor in the production of shoot cultures from any tree in these experiments.

Phytohormone treatment effects. Although increasing BAP levels did not influence the *in vitro* performance of trees #2, #3, and #5 relative to trees #1 and #4, the number of shoots which were obtained from explants of trees #2 and #5 was maximized at the highest BAP concentration tested (Table 4). Shoots from trees #2 and #3 appeared vitrified in all treatments. Increasing BAP levels further would seem unlikely to eliminate the problem of propagating trees #2 and #3, given the increased tissue vitrification which was associated with high BAP levels in cultures initiated with explants from juvenile black locust (unpublished data), and

in other tree species (von Arnold and Eriksson, 1984). Explants from tree #5, however, produced extensive callus at the lower BAP concentrations, and produced non-vitrified shoots at the higher BAP levels. This may reflect a higher effective level of endogenous auxin in this tree, possibly associated with the physiological changes that occur prior to the initiation of shoot elongation in the spring. If the five trees which were used in these experiments represent a random sample, buds obtained from most mature black locust trees should be capable of proliferating on MS medium containing 0.32 or 3.2 uM BAP.

In the rooting experiment, the average root diameter was less in treatments containing IBA alone than in treatments which contained NAA. Root induction also took longer in medium containing IBA alone. While these data alone indicate that NAA increases the efficiency with which black locust shoots can be rooted, the roots induced in the presence of NAA were not as firmly attached to the shoots as those produced in medium with IBA alone. This may have been due to the presence of callus tissue at the root-shoot junction. A root initiation medium which contained lower strength basal salts concentration and IBA alone was also selected for root induction on micropropagated shoots of *R. pseudoacacia* cv. 'Jaszkiseri' (Barghchi, 1987), and induced the best *in vitro* rooting of micropropagated shoots from *Malus* sp. (Dunstan and Turner, 1984).

The medium that contained 0.5 uM of each auxin induced a rooting response which was generally intermediate between the treatments containing each auxin alone. This suggested an additive effect of the auxins on the number of shoots which produce roots, and the speed at which rooting takes place (Figure 6). This would be expected if NAA and IBA stimulate similar biochemical processes in the plant, but NAA to a greater extent than IBA. The generality of this phenomenon across all genotypes of black locust is not known.

Explant source-specific responses. The shoot proliferation of trees #1 and #4 relative to the other trees, across all BAP levels, indicated that the superior performance of these two trees in culture was not simply due to optimization of the phytohormone concentrations in the medium for each tree. Although peak responses were observed for the promotion of shoot proliferation and elongation for trees #1 and #4 (Table 2), their rank did not change relative to the other trees. This suggests that the positive response of the explants from trees #1 and #4 to this tissue culture system was due to factors inherent in those particular trees.

Those inherent differences were also apparent when the rhizogenic capacity of the shoots from trees #1 and #4 was compared (Figure 6). Two to three times as many shoot explants from tree #1 produced roots compared to tree #4. The differences between explant sources with respect to

percent rooting were much larger than the auxin treatment differences which were observed. In an independent rooting experiment in which 1.0 uM IBA was used in the medium (Han *et al.* submitted), shoots used as explants were obtained from callus regenerants of trees #1 and #4. Forty out of 72 (56%) of the shoots from tree #1 produced roots after four weeks, whereas 3/16 (19%) of the shoots derived from tree #4 rooted. Shoot proliferation from the shoots derived from callus of tree #1 was also significantly higher than for tree #4. These results are what would be predicted from the experiments described in this paper. The reduction in rooting percentage for both clones in the latter experiment could have been due to the smaller container size and volume of medium which was used.

The basis for the tree-specific responses among the five trees could not be elucidated in these experiments due to the confounding of genetic effects, physiological and environmental factors such as site, and differences in tree age. The persistence of the explant source-specific differences in the shoots that were regenerated from callus (Han *et al.*, submitted) suggests that the genetic or epigenetic factor(s) which form the basis for the differential responses were not altered during morphogenesis. Since all the trees which were tested are mature, the inheritance of genes that affect *in vitro* performance in black locust trees can now be tested.

There has been discussion in the literature regarding pronounced differences due to genotype with respect to performance in tissue culture systems. Some work has indicated that explant pretreatments (Gharyal and Maheshwari, 1983; Franclet, 1985) and the use of different phytohormones in media (Komatsuda and Ohyama, 1988) can override differential responses due to genotype differences. Our data indicate that different rooting media did not override clonal differences (Figure 6), and the clonal differences persisted even after regeneration of shoots from callus (which could be considered a pretreatment; Han *et al.*, submitted). However, the possibility that the shoot proliferation media were not optimized for trees #2, #3, #4, and #5 could never be eliminated unless such a medium was discovered. While cultural treatments can profoundly affect explant performance, it seems reasonable to postulate that some genotypes are inherently poor performers *in vitro*, possibly due to low tolerance to the high water potentials that exist in culture.

Furthermore, if amenability to tissue culture is even partly due to genetic differences among the trees, these results have important practical implications for breeding programs which plan to use micropropagation to replicate mature trees which have been tested, and are known to have elite genotypes. These results indicate that *in vitro* performance should be considered as a component of the selection strategy in the early stages of the breeding

program. This will ensure that most genotypes included in the breeding pool are amenable to *in vitro* manipulation.

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CHAPTER 2

Detection and analysis of T-DNA in crown gall tumors of *Robinia pseudoacacia*.

ABSTRACT

Five *Agrobacterium tumefaciens* strains, and one *Agrobacterium rhizogenes* strain, incited tumors on *Robinia pseudoacacia* L. (black locust) tissues that were inoculated *in vitro*. Five different phytohormone-independent tumors, incited by *Agrobacterium tumefaciens* strains A6, A348, A274, and A208, were tested for the presence of T-DNA using Southern analysis. DNA sequences which hybridized to cloned T_L-region probes (from pTiA6) were detected in all the tumors, and the results were consistent with the expectation that the respective T-DNAs would be integrated into the black locust genome. Some tumors tested negative for the presence of octopine (tumors incited by A6, A348 and A274) or nopaline (tumor incited by A208). All these tumors had T-DNAs which were truncated on the right ends, *i.e.*, missing the region in which those particular opine synthetic loci were present on the Ti plasmid.

INTRODUCTION

Agrobacterium-mediated gene transfer methods utilize the natural gene transfer apparatus of *Agrobacterium tumefaciens* or *A. rhizogenes*, closely related plant pathogens which incite crown gall tumor and hairy root diseases, respectively (reviewed in Zambryski *et al.*, 1989). Pathogenic *A. tumefaciens* strains deliver DNA (called T-DNA, for transferred DNA) into cells of plant hosts (reviewed in Koukalikova-Nicola *et al.*, 1987; Zambryski, 1988; Zambryski *et al.*, 1989), after which it is integrated into the plant nuclear genome (Thomashow *et al.*, 1980; Zambryski *et al.*, 1982). Native T-DNA contains oncogenes which encode enzymes directly involved in the auxin (Schroder *et al.*, 1984; Thomashow *et al.*, 1984, 1986) and cytokinin (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984) biosynthetic pathways. This forms the basis for the phytohormone-independent growth which is characteristic of crown gall tumor cells. In addition, crown gall cells also produce one or more opines (reviewed in Tempe *et al.*, 1984) which the bacterium can utilize as its sole carbon and nitrogen source.

Agrobacterium can also be used as a vector to deliver altered T-DNAs into plant cells (Zambryski *et al.*, 1983). Once developed for black locust, this technique will be used to augment tree improvement efforts, and to investigate fundamental aspects of gene regulation in *Robinia pseudoacacia* L. (black locust). In addition to its international economic importance (Keresztezi, 1980), this

leguminous tree species has a small genome (2.7 pg per diploid cell; Singh and Siminovitch, 1976), a short generation interval (often 4 years from seed in plantations; J. Hanover, pers. comm.), and exhibits rapid vegetative growth after establishment in the greenhouse or field. In addition, black locust appears to be amenable to tissue culture (Brown and Sommer, 1982; Chalupa, 1983, 1987; Davis and Keathley, 1985, 1987; Barghchi, 1987; Han and Keathley, submitted; Han *et al.*, submitted), in contrast to most woody legumes, which have proven recalcitrant to growth *in vitro*.

Since no reports could be found which suggested *R. pseudoacacia* was a host for *A. tumefaciens* or *A. rhizogenes*, the first objective of the experiments described in this paper was to determine if phytohormone independent tumor growth could be incited on black locust tissues. The second objective was to examine DNA from these putative tumor cells to determine if sequences which hybridized to cloned T-region probes could be detected, and to obtain evidence for integration of those sequences into the *R. pseudoacacia* genome. The third objective of the present study was to develop partial physical maps of the T-DNA constructs in order to address the question of why opines were not detected in some of the tumor tissues. To our knowledge, this report is the first to present physical evidence for stable maintenance of foreign DNA in tissues of a woody legume.

MATERIALS AND METHODS

Black locust seeds were placed in boiling water (20 sec), transferred to 5% NaOCl (5-10 sec), placed on Whatmann #1 filter paper (11.0 cm diameter) to dry, and allowed to germinate *in vitro* on MS salts and vitamins medium (Murashige and Skoog, 1962) lacking phytohormones and containing 5 g/l sucrose. After 20 days, hypocotyl segments (5 mm long, oriented vertically) or excised cotyledons were placed on MS medium lacking phytohormones (MSO).

Hypocotyl segments were inoculated by placing 1 ul from an overnight *Agrobacterium* suspension (3 ml LB medium, 30^{o}C, A_{600}= 1.5) on the apex. After 3 days, the apices of the hypocotyl segments were excised and placed on MSO containing 300 ug/ml cefotaxime (Calbiochem) and 300 ug/ml carbenicillin (Sigma). The tissue was subsequently maintained on MSO containing 500 ug/ml of both antibiotics.

Cotyledons were inoculated with bacteria taken directly from a single colony grown for 2 days on agar-solidified LB at 30^{o} C. A needle was placed in the colony, and then four parallel wound regions 1 cm long were generated by gently passing the needle tip across the upper surface of the cotyledon. After 4 weeks, individual tumors were excised from the cotyledon surface, and maintained on MS basal medium with antibiotics as described for the tumors induced on hypocotyls.

To assay for the presence of octopine or nopaline, gall tissues were manually ground with a glass rod in

microcentrifuge tubes with 1 volume 95% ethanol, centrifuged 5 min, and 3 ul of the supernatant was spotted per lane on Whatmann 3MM paper. Electrophoresis and staining with phenanthrenequinone were performed according to previously described methods (Otten and Schilperoort, 1978).

To isolate high molecular weight plant DNA, gall and callus tissues were ground to a fine powder in liquid nitrogen, the powder was suspended in 3-5 volumes of extraction buffer containing 50 mM Tris (pH 8.0), 10 mM EDTA, 1 M NaCl, 0.02% Sarkosyl, 10 mM 2-mercaptoethanol, 3 mM cysteine, and 1 mM ascorbic acid, and the suspension was filtered through 100 micron nylon mesh. After extraction with 1 volume of phenol, and 1 volume of chloroform: isoamyl alcohol (25:1), nucleic acids were precipitated from the aqueous phase (Maniatis *et al.*, 1982) and pelleted at 12,000 x g for 15 min. The pellet was dissolved in TE buffer, after which solid CsCl and bisbenzimide (final concentration 20 ug/ml; Calbiochem) were added to achieve a refractive index of 1.3955 prior to equilibrium density centrifugation.

Total *Agrobacterium* DNA was isolated as in Buchholz and Thomashow (1984), and DNA samples were digested with restriction endonucleases *Eco*RI, *Hin*dIII (obtained from Boehringer Mannheim Biochemicals), *Bam*HI, *Pst*I, *Sal*I, or *Sst*II (obtained from Bethesda Research Labs). Plant DNA (7 ug) and bacterial DNA (50 ng) samples were size fractionated by electrophoresis in 0.8% agarose gels, and the gels were washed according to the method of Wahl *et al.* (1979). DNA

was then blotted to nitrocellulose according to previously described methods (Southern, 1975, as described in Maniatis *et al.*, 1982). Purified plasmid DNA was obtained using the method of Birnboim and Doly (1979). Specific restriction fragments to be used as probes were isolated using NA-45 paper (obtained from Schleicher & Schuell). Probe DNA (50 to 500 ng) was radioactively labelled using a random primed labelling kit obtained from Boehringer Mannheim Biochemicals with ^{32}P-dCTP (3000 Ci/mmol; New England Nuclear). Hybridization, washing, and autoradiography were performed as described in Thomashow *et al.* (1980), except that probe DNA and salmon DNA were added to the hybridization solution to final concentrations of 12.5 ng/ml and 100 ug/ml, respectively.

RESULTS AND DISCUSSION

Six of the *Agrobacterium* strains incited tumors which were visible 4 weeks after inoculation (Table 1). *A. rhizogenes* strain A4 incited tumors which produced roots; *A. tumefaciens* strains incited unorganized tumors. A lower percentage of hypocotyl explants (16%) formed tumors than cotyledons explants (31%) when inoculated with *A. tumefaciens*. Inoculation with R1000 and the avirulent strains A136 (this strain has been cured of the Ti plasmid) and A6.1d$_3$ (this strain carries a mutation at *psc*A, a chromosomal locus required for virulence) did not result in tumor formation on any black locust explant. With the

exception of A136 and A6.1d$_3$, all strains formed tumors on *Kalanchoe diagremontia* stems. The apparent inability of R1000 to form tumors on black locust explants may have simply been an artifact of the small sample size.

A total of four rapidly growing phytohormone-independent tumor lines (two octopine-negative, incited by A274 and A348; one octopine-positive, incited by A6; one nopaline-negative, incited by A208) were selected for further analysis. The T-regions of pTiA6 (in A6 and A348) and pTiB$_6$806 (in A274) generate identical *Bam*HI, *Hin*dIII, and *Eco*RI restriction maps (Sciaky *et al.*, 1978; Thomashow *et al.*, 1980; Davis, unpublished), so DNA from the tumor lines incited by octopine strains was compared directly to A6 bacterial DNA on Southern blots.

T-DNA sequences appeared to be integrated into the black locust genome in all three tumor lines which were incited by octopine-type strains. Hybridization was detected between the tumor DNA digested with *Bam*HI and the 7.8 kb *Bam*HI fragment from the T$_L$-region of pTiA6 (Bam8; see Figure 1) used as a radiolabelled probe (Figure 2). The bands in lanes 1-3 are due to hybridization of the probe to fragments of different size than the bacterial Bam8 fragment (lane 5). Since Bam8 contains the left border sequence (Figure 1), these results would be expected if integration into the black locust genome had occurred. *Hin*dIII fragment 1, which contains the right border sequence, was also altered in all three tumors.

Table 1. Strains of *Agrobacterium* spp. tested for virulence on black locust explants, and the number of explants which formed at least one tumor 4 weeks after inoculation.

Strain	Relevant plasmid[1]	Explant source	No. of explants	No. explants forming tumors
A6	pTiA6 (1)	hypocotyl	20	2
		cotyledon	4	2
A348	pTiA6 (1)	hypocotyl	12	1
		cotyledon	12	5
A274	pTiB$_6$806 (1)	hypocotyl	12	0
		cotyledon	12	1
A208	pTiT37 (2)	hypocotyl	20	6
		cotyledon	8	3
A281	pTiBo542 (3)	hypocotyl	11	3
A4	pRiA4a,b (4)	hypocotyl	11	2
R1000	pRiA4b (4)	hypocotyl	16	0
		cotyledon	4	0
A136	(cured) (5)	hypocotyl	12	0
A6.1d$_3$	pTiA6 (6)	hypocotyl	4	0
		cotyledon	12	0

[1] The following references contain maps of the T-region or a description of the bacterium: (1) Thomashow *et al.*, 1980; (2) Joos *et al.*, 1983; (3) Hood *et al.*, 1987; (4) Huffman *et al.*, 1984; (5) Watson *et al.*, 1975; (6) Thomashow *et al.*, 1987.

Figure 1. Physical and genetic maps of the T_L-region of pTiA6, and a partial map of T-DNA present in crown gall tumors incited by inoculation of *Agrobacterium tumefaciens* strains A348 and A274 on *Robinia pseudoacacia*. Restriction fragment nomenclature is according to Thomashow *et al.* (1980) and Nester *et al.* (1984). Fragments used as probes in this study are designated by bold underlines. The border sequences (flags) for the T_L-region were localized using the map of Gielen *et al.* (1984). Oncogenic loci, onc; octopine synthase locus, ocs.

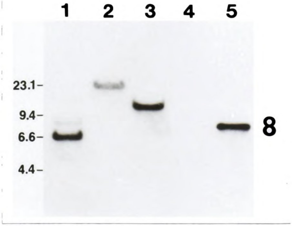

Figure 2. Autoradiograph of a Southern filter with *Bam*HI-digested black locust tumor and callus DNA, and A6 bacterial DNA, after hybridization with ^{32}P-labelled *Bam*HI fragment 8 from pTiA6. Lanes: 1) tumor incited by A6; 2) tumor incited by A348; 3) tumor incited by A274; 4) callus; 5) A6 bacteria. Molecular size standards (kb) are shown along the left margin; Bam8 is labelled along the right margin.

The observed hybridization of the probe to the gall DNA was not due to contamination by *A. tumefaciens* in the gall tissues, since production of the bands seen in Figure 2 would have required extensive alteration of the T-region in the bacteria. In addition, no bacteria were detected when the tumor tissue was streaked on LB medium, and hybridization was only observed between bacterial DNA samples and a pTiA6 *vir* region probe on a Southern filter containing both tumor and bacterial DNA (*Sal*I fragments approx. 5 and 5.5 kb in size, containing *vir*C and *vir*D loci, were labelled as a probe after isolation from pSM304, a plasmid described in Stachel *et al.*, 1986).

The physical maps of T-DNA present in the tumors incited by octopine-type strains (except A6, which appeared to contain many partial T-DNA copies, and was therefore not mapped) are also shown in Figure 1. Left T-DNA/plant DNA junction sites mapped within the expected HindY fragment (Bevan and Chilton, 1982; *Hin*dIII fragment Y corresponds to *Hin*dIII fragment 18c of pTiAch5) in all three tumors incited by the octopine strains. This conclusion was reached because *Hin*dIII fragment X was intact in all three lines, whereas *Hin*dIII fragment Y was absent (and replaced by a fragment of different size). Physical mapping of T_R-DNA (Thomashow *et al.*, 1980) was not needed to accomplish the objectives of this study.

In order to meet the third objective of this study (to determine if the opine-negative phenotype of certain tumor

lines could be explained), the right extreme of the T-DNA was mapped in the two octopine-negative tumor lines using Eco7 and Bam29 fragments as probes (Figure 3). This was done because the right ends of the T_L-region of pTiA6 (Figure 1), and the T-region of pTiT37 (Figure 5), contain the octopine and nopaline synthetic loci, respectively.

A348 tumor: The absence of an intact *Eco*RI fragment 7 in this tumor (Figure 3A, lane 2) suggested that one end of the Eco7 fragment had been altered by plant DNA, or that a deletion had occurred. *Bam*HI fragments 29 (Figure 3C, lane 2) and 29' (Figure 3B, lane 2), and *Hin*dIII fragments I and II (data not shown), appeared intact. However, *Bam*HI fragment 19 was missing, and a shorter fragment was present in the tumor DNA (Figure 3B, lane 2). I conclude that the T-DNA insert in this tumor line was truncated on the right end, and this, in turn, indicated that the *ocs* locus was absent from this tumor. This would be consistent with an inability to detect octopine in the tumor tissue (Figure 4, lane 3.)

A274 tumor: A 4 kb fragment was observed when a filter with *Hin*dIII-digested tumor DNA was probed with Eco7. The presence of this 4 kb *Hin*dIII fragment suggested that the right end of the T_L-DNA may have been truncated. This hypothesis was supported by the observation that *Bam*HI fragments 29 and 29' were intact, whereas *Bam*HI fragment 19 was absent (Figure 3B, lane 3). Consistent with the

Figure 3. Autoradiographs of Southern filters with black locust tumor and callus DNA, and A6 bacterial DNA, after hybridization with ^{32}P-labelled restriction fragments from the T_L-region of pTiA6. Lanes: 1) A6-incited tumor; 2) A348-incited tumor; 3) A274-incited tumor; 4) callus; 5) A6 bacteria. Molecular size standards (kb) are shown along the left margin; T-region fragments are labelled along the right margin.

A. DNA was digested with *Eco*RI, and *Eco*RI fragment 7 was used as a probe.

B. DNA was digested with *Bam*HI, and *Eco*RI fragment 7 was used as a probe.

C. DNA was digested with *Bam*HI, and *Bam*HI fragment 29 was used as a probe.

physical map, octopine was not detected in this tumor (Figure 4, lane 4).

Faint hybridization was sometimes detected in the lanes which contained normal callus DNA (Figure 3B, lane 4). Normal black locust DNA may therefore possess some region of sequence similarity to the T-region probes used in this study. Faint hybridization of *Hin*dIII fragment 1 to normal tobacco callus DNA has also been reported (Thomashow *et al.*, 1980).

A208 tumor: DNA from the nopaline-negative tumor line was digested with *Hin*dIII, or *Eco*RI, and Eco7 was used as a probe. The nopaline-negative tumor had an intact *Hin*dIII fragment 22, while *Hin*dIII fragment 31 was not present. *Eco*RI fragment 1 was not intact in this tumor; rather, a

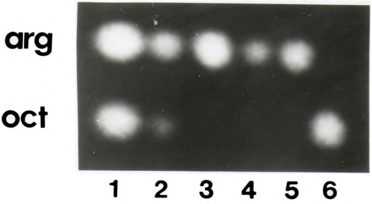

Figure 4. Paper electrophoretogram of ethanol-extracted crown gall tumors incited on black locust by *Agrobacterium tumefaciens* strains A6, A348, and A274. Lanes: 1) authentic octopine (oct) and arginine (arg; 2 ug each); 2) A6 tumor; 3) A348 tumor; 4) A274 tumor; 5) callus; 6) authentic octopine (2 ug). 366 nm UV illumination.

single fragment 6 kb in size hybridized to the probe. These data suggest that, in a manner analagous to the octopine-negative tumors, the *nos* locus was not present in this tumor (Figure 5).

Single T-DNA-plant DNA border fragments were observed in the A348, A274, and A208 tumor DNA (although long overexposures of Southern filters revealed minor bands in the A274 tumor). Since the tumors tested in this study were not cloned from single cells, one might have expected that the tissues would be composed of cells which differed widely with respect to their T-DNA complement (*i.e.*, had complex restriction patterns) similar to that observed in the A6 tumor. Hood *et al.* (1987) identified single border fragments during T-DNA mapping experiments performed on uncloned soybean and alfalfa tumors which were incited by *Agrobacterium tumefaciens* strain A281, and speculated that there may have been *in vitro* selection for the cells which contained the observed constructs. In a similar fashion, selective proliferation of black locust cells which contained particular T-DNA constructs could have given rise to the results reported here. For example, in the case of the A348 and A274 tumors, *onc*-positive / *ocs*-negative cells could have selectively proliferated. Given this scenario, then *onc*-negative or *ocs*-positive cells could be selected against in the tumor tissue.

If there was selection against the octopine-positive tumor cells, then one might expect that differences in the

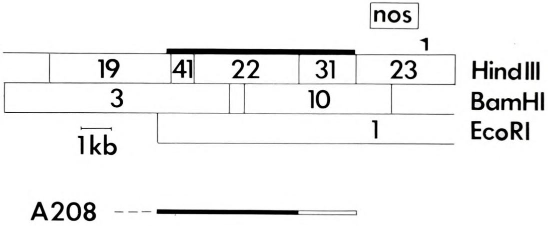

Figure 5. *Hin*dIII and *Eco*RI restriction maps of the right end of the pTiT37 T-region, and a partial physical map of the T-DNA in the nopaline-negative tumor line. Restriction fragment nomenclature is according to Joos *et al.* (1983).

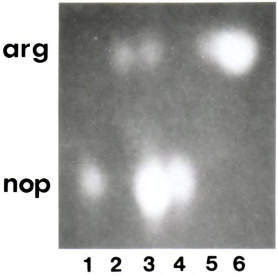

Figure 6. Paper electrophoretogram of ethanol-extracted crown gall tumors incited by *Agrobacterium tumefaciens* strain A208. Lanes; 1) nop$^+$ tumor incited on *Kalanchoe* stem; 2) nop$^-$ tumor from black locust; 3) nop$^+$ tumor from black locust; 4) authentic nopaline (2 ug); 5) callus; 6) authentic arginine (2 ug). 366 nm UV illumination.

relative growth rates of the A6 tumor and the A348 or A274 tumors would be apparent. These differences were not observed. In addition, the fresh weight of normal black locust tissues which were cultured on medium which contained 10 mM octopine did not differ from tissue cultured on medium lacking octopine (Table 2). These data argue against the suggestion that octopine-producing cells were selected against in the tumors incited on black locust. An alternative possibility is that the production of an actively dividing tumor cell may be a relatively rare event after inoculation of black locust tissues with *Agrobacterium* (only 21% of the explants formed a tumor; Table 1). As such, perhaps one, or a very few transformed cells normally give rise to a tumor which continues to proliferate. I

Table 2. Average weight, and frequency of shoot production of calli from black locust hypocotyl explants which were cultured on callus induction medium (MS with 5 uM NAA and 5 uM BAP) in the presence of 0, 0.1, or 10 mM octopine. Individual hypocotyls (seedlings) were randomized across octopine treatment levels.

	Treatment (mM octopine)			
	0	0.1	10	F[1]
Avg. callus wt. (± S.E.)	1.64[2] (0.79)	1.65 (0.82)	1.40 (0.70)	ns
Freq. explants with shoots	0.21	0.23	0.17	ns

[1] F-test from analysis of variance; ns, not significant at alpha=.05.

[2] Observations per datum: 29 (0 mM), 32 (0.1 mM), and 36 (10 mM).

would expect that screening a large number of tumors would yield a wide range of intact, truncated, and rearranged T-DNA constructs, all of which have been demonstrated to occur in plants previously identified as hosts for *Agrobacterium* (Gielen *et al.*, 1984; Simpson *et al.*, 1986).

The results of these experiments demonstrate that *A. tumefaciens* can introduce DNA into the *R. pseudoacacia* genome when the plant tissues are inoculated *in vitro*, and that the T-DNA is stably maintained in the plant cells. The use of *A. rhizogenes*-based vectors should also be possible with this species (Table 1). These results suggest that failed attempts to regenerate transgenic shoots (Davis, unpublished) by the use of a leaf disk regeneration protocol (Davis and Keathley, 1985) were probably not due to the inability of the vector to deliver T-DNA into the cells.

Even in the absence of a useful protocol for regeneration of transgenic shoots from leaf disks, experiments designed to introduce selectable marker loci into black locust cells can now be initiated. The goal of these investigations will be to produce callus from transgenic cells. Results of recent experiments (Davis and Keathley, 1987; Han *et al.*, in review) suggest that certain black locust trees may be particularly amenable to *in vitro* culture and regeneration. Studies of the *in vitro* responses of herbaceous legumes (*e.g.*, Bingham *et al.*, 1975; Malmberg, 1979; Komatsuda and Ohyama, 1988) have indicated that a significant component of such responses is genetic. This

suggests that the probability for successful production of transgenic callus (and subsequent regeneration into plantlets) in highly heterozygous woody legumes such as black locust may be greatly enhanced by initial efforts to screen germplasm.

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CHAPTER 3

Induction of transgenic callus from explants of *Robinia pseudoacacia*

ABSTRACT

Cotyledons, hypocotyls, and stem segments were inoculated with *Agrobacterium* strains that had loci encoding selectable markers in the T-region. One kanamycin resistant callus was obtained after inoculation of cotyledons with an *A. tumefaciens* strain carrying a binary vector (pGA472; An *et al.*, 1985). A second kanamycin resistant callus, which also produced nopaline, was obtained after inoculation of stem segments with a disarmed cointegrate vector (pGV3850HPT::pKU2; Baker *et al.*, 1987). Southern analysis of each of these callus lines revealed that the expected T-DNA was present in the tissues, and the sequences were integrated into the black locust genome. Evidence was also obtained which suggested that plant genotype had an important effect on the production of transgenic callus.

INTRODUCTION

Examinations of several herbaceous legumes by various investigators (Bingham *et al.*, 1975; Reisch and Bingham, 1980; Keyes *et al.*, 1980; Komatsuda and Ohyama, 1988) have led to the conclusion that significant genetic variation exists in this taxon of plants with respect to *in vitro* responsiveness of their tissues. It is possible that similar variability exists in black locust, since high levels of genetic heterogeneity have been observed in black locust populations (Surles *et al.*, 1989a) maintained in part due to a high degree of outcrossing (Surles *et al.*, 1989b). Genotype-specific effects in black locust may be reflected in the tree- or family-specific responses which have been observed with respect to shoot proliferation (Chapter 1; Davis and Keathley, 1987), callus induction and regeneration (Han and Keathley, in review; Han *et al.*, in review), rooting of micropropagated shoots (Chapter 1; Han *et al.*, in review), and rooting of hardwood cuttings (Prentice, 1987).

In Chapter 2, physical evidence was presented that stable T-DNA transfer from *Agrobacterium* to black locust cells occurred after inoculation with 4 different *A. tumefaciens* strains. The overall objective of the experiments described in this chapter was to examine the differential responses of various black locust genotypes to the induction of transgenic callus growth from explants, after inoculation of those explants with modified *Agrobacterium* strains. These modified *Agrobacterium* strains

contain dominant selectable markers in the T-region, such that genes encoding antibiotic resistance should be transferred into susceptible black locust cells. The specific objectives of these experiments were to obtain callus that proliferates on medium containing a selective agent that is toxic to normal cells, to obtain evidence that the cells are indeed transgenic, and to assess the importance of plant genotype on the formation of transgenic tissues.

MATERIALS AND METHODS

Bacterial strains. *Agrobacterium* strain pGV3850HPT::pKU2 (Baker *et al.*, 1987) is a disarmed cointegrate vector derived from pGV3850 (Zambryski *et al.*, 1984). The physical integrity of both *npt*II and *hpt* loci was verified by Southern analysis of DNA from broth cultures initiated from single kanamycin resistant (25 ug/ml) colonies. The *nos* locus is present on the right end of the T-region in this strain.

Two binary strains were tested in these experiments, although only one of them, A281 (pGA472) incited growth of transgenic callus. A281 (pGA472) possesses the shuttle vector pGA472 in the "supervirulent" background of *A. tumefaciens* strain A281 (An *et al.*, 1985). This strain has a chimeric *npt*II construct (including the *nos* promoter and polyadenylation signals) which serves as a kanamycin resistance marker for maintenance in *Agrobacterium*, and also

as a plant selectable marker. The second binary strain, A281 (pCIB715), has the shuttle vector pCIB715 (Rothstein *et al.*, 1987) in the *A. tumefaciens* strain A281 background. This strain was constructed by mating *A. tumefaciens* strain A281 with *E. coli* strain S17-1 containing pCIB715 on LB agar plates. *E. coli* S17-1 has the RP4 mobilizing functions integrated into the bacterial chromosome (Simon *et al.*, 1983). Exconjugants were selected on LB containing rifampicin (5 ug/ml) and kanamycin (25 ug/ml). The presence of the shuttle vector, T-region and *vir*-region in antibiotic resistant bacteria was verified by Southern analysis of DNA isolated from broth cultures initiated from single colonies.

A136 (Watson *et al.*, 1975), which lacks a Ti plasmid, or A6.1d$_3$ (Thomashow *et al.*, 1987), which lacks a chromosomal locus required for virulence, were used as avirulent control strains for the experiments.

Plant materials. To produce seedlings for inoculation of hypocotyl or cotyledon explants, seeds collected from various trees in the East Lansing, MI area were boiled in water (20 sec), immersed in 5.25% NaOCl (10 sec), placed on filter paper to dry, and then cultured on 5 ml of agar-water (0.8% w/v) in 18x150 mm culture tubes for subsequent germination. After 3 weeks, seedlings were randomly selected for inoculation with bacteria.

Shoot cultures originally derived from a 21-year old tree (tree #1; described in Davis and Keathley, 1987) were

maintained by monthly subculture on shoot proliferation medium (10 ml MS medium supplemented with 0.5 uM BAP in 25x150 mm culture tubes).

To produce clonal lines of individual seedlings for use in stem inoculation studies, seeds were obtained from 4 different trees which were found to differ in their response to bud culture (Davis and Keathley, 1987), and a fifth group of seeds was collected from randomly selected trees in the East Lansing, MI area. Seeds from each family were surface sterilized and placed on agar-water medium as described above. After 3 weeks of growth, cotyledonary nodes were excised from 20 randomly selected seedlings per family. These were placed on shoot proliferation medium for initiation of shoot cultures. When the cotyledonary node is used as an explant, at least three shoot apices normally begin to elongate and proliferate; the epicotyl, and a minimum of two latent shoots from the node itself (Davis, unpublished). After 6 weeks, the shoots were harvested for inoculation with bacteria. One shoot from each clone was transferred to fresh shoot proliferation medium in order to maintain each clonal line.

Wounding and inoculation procedures. Cotyledons were removed from seedlings, wounded on the upper surface by gently slicing the epidermis 5-6 times with a scalpel blade, and then placed on callus induction medium (wounded side up). Two to three ul of overnight broth cultures of A281 (pGA472), A281 (pCIB715), or pGV3850HPT::pKU2 were placed on

wounded cotyledons, and the droplet was spread evenly over the cotyledons with a bacterial loop. Inoculations using *A. tumefaciens* A281 (pGA472) onto cotyledons were performed prior to the experiment in which the other two strains were tested. Prior to inoculation with these other two strains, A281 (pCIB715) and pGV3850HPT::pKU2, hypocotyls were sectioned into pieces 2-4 mm in length, and placed upright on agar-solidified callus induction medium. Cotyledon inoculations were performed as described above.

To prepare stems from shoot cultures for inoculation with bacteria, stems were cut into segments 2-3 cm in length, and wounded by scraping along the entire length of the segment with a scalpel blade. Each segment was then placed on 25 ml of agar-solidified (0.8%) callus induction medium (MS salts and vitamins supplemented with 5 uM of both NAA and BAP) in 100x15 mm plastic Petri dishes. This callus induction medium was selected based on the experiments of Han *et al.* (manuscript in review). Mature tree #1 stem segments were then inoculated with 5-10 ul of an overnight broth culture of *Agrobacterium* strain pGV3850HPT::pKU2 with or without acetosyringone (100 uM, incubated for 2 hr prior to inoculations), or the binary vector A281 (pCIB715). The segments were left for 10 hr in the dark at 25^{o}C.

Stem segments from clonal material derived from seedlings were inoculated with 2-3 ul of a liquid suspension of pGV3850HPT::pKU2 (bacteria from an overnight broth

culture were pelleted, and resuspended in MS lacking phytohormones; $A_{600} = 1.5$). The petri dishes were sealed with Parafilm$^{(R)}$, and left in the dark for 3 days (25°C).

Culture on selective medium. The levels of hygromycin (20 ug/ml) and kanamycin (100 ug/ml) used for transgenic cell selection were chosen based on the previous experiments of Baker *et al.* (1987) and Rothstein *et al.* (1988). To ensure that normal tissues of *R. pseudoacacia* would not proliferate on callus induction medium which contained these levels of antibiotic, stem segments (1-2 mm in length) were excised from cloned seedlings, and placed on medium containing 5, 10, 15, 20, or 30 ug/ml hygromycin, or 25, 50, 100, or 150 ug/ml kanamycin. At least 8 explants were cultured per treatment, and each explant was weighed after 4 weeks of culture.

Cotyledon and hypocotyl explants which had been inoculated with bacteria, were transferred to callus induction medium supplemented with 500 ug/ml of both cefotaxime (Calbiochem) and carbenicillin (Sigma), and either kanamycin or hygromycin. After 4 and 6 weeks on this medium (with a weekly transfer interval), data were collected on the number of live calli.

Stem segments which had been inoculated with bacteria were transferred to callus induction medium supplemented with the same antibiotics as described above. After three weeks on this medium (with a weekly transfer interval), the segments were sectioned into 3-5 mm pieces, and transferred

to fresh medium. For the next month, data were collected weekly on the number of live calli.

DNA isolation and analysis. DNA isolation, digestion, electrophoresis, and Southern analysis were performed according to previously described methods (see Chapter 2). DNA fragments used as probes were isolated from *E. coli* S17-1 (pCIB715) digested with *Bam*HI (*hpt* locus) or *E. coli* S17-1 (pCIB10) digested with *Pst*I (*npt*II locus).

RESULTS AND DISCUSSION

Binary strains of *A. tumefaciens* are often preferred for introduction of foreign DNA into plant tissues due to their ease of construction compared with cointegrate vectors (Klee *et al.*, 1987). The binary strain A281 (pGA472) was tested for its ability to induce formation of kanamycin resistant callus on cotyledons in a preliminary experiment. One callus resistant to 100 ug/ml kanamycin was obtained. Sequences related to *npt*II were detected in DNA from this tissue, but not in normal callus DNA (Figure 1). These sequences appeared to be integrated into the *R. pseudoacacia* genome, since the fragment that hybridized to the *npt*II probe in the DNA from kanamycin resistant callus was > 3 kb in size. This conclusion was reached because the distance between the unique *Hin*dIII site of pGA470 (and pGA472) and the right border is approximately 2.3 kb, the same size as the fragment generated after digestion of the plasmid DNA with *Hin*dIII and *Sst*II used as positive controls (lanes 4

and 5). Contamination by the bacterium does not account for the observed hybridization, since a single 15.6 kb band (the size of pGA472) was not observed in the lane containing the kanamycin resistant callus DNA.

Since physical evidence for stable introduction of *npt*II sequences into *R. pseudoacacia* had been obtained, and a protocol for regeneration of shoots from *R. pseudoacacia* had been developed (Davis and Keathley, 1985), a series of leaf disk inoculation experiments similar to the study of Horsch *et al.* (1985) were initiated. The results of these experiments are not reported in this chapter, since no kanamycin resistant calli or shoots were obtained. It was decided that other factors should be explored in an effort to obtain transgenic tissues.

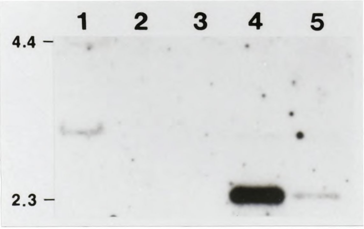

Figure 1. Southern blot showing hybridization of probe (1.9 kb *Hin*dIII-*Pst*I fragment from pGA470 (An *et al.*, 1985), containing the 3' end of the *npt*II coding region) to DNA from kanamycin resistant callus. Lanes: 1) kanamycin resistant callus; 2) normal callus; 3) *A. tumefaciens* strain A281; 4) A281 (pGA472); 5) purified pGA470. DNA samples were digested with *Hin*dIII alone (lanes 1 and 2), or in combination with *Sst*II (lanes 3-5).

A factor that could profoundly influence transgenic callus production is the reporter gene used. Hygromycin was chosen as an alternative selective agent to kanamycin to test on black locust for two reasons. Hygromycin has proven a superior alternative to kanamycin as a selective agent for production of transgenic callus in *Arabidopsis thaliana* (Lloyd *et al.*, 1986) and *Populus* sp. (M. Gordon, personal communication). In addition, two *Agrobacterium* strains were available that possessed both *npt*II and *hpt* loci within the T-region (described in Materials and Methods).

The proliferation of normal black locust tissues into callus was reduced by the presence of either kanamycin or hygromycin in the callus induction medium (Figure 2). The average callus weight after the 4 week culture period ranged from 615 mg (no added antibiotic) to less than 25 mg. The results of this experiment showed that the levels of hygromycin (20 ug/ml) and kanamycin (100 ug/ml) chosen for use in these experiments were toxic to normal tissues of *R. pseudoacacia*.

Stems from shoot cultures derived from a mature tree which responded favorably to shoot proliferation medium, were also inoculated with pGV3850HPT::pKU2 or A281 (pCIB715). No hygromycin resistant or kanamycin resistant calli grew from these explants (Table 1). The addition of acetosyringone to the bacterial broth culture prior to inoculation had no effect on subsequent callus formation, which suggests that the barrier to transgenic callus

production was not due to a lack of *vir* induction in the bacteria. This is further supported by the observation that tumor formation was observed when *A. tumefaciens* strain A208 was inoculated on stems of this genotype in previous experiments (A208 differs from the cointegrate vector in the T-region only). Normal stem tissues from this genotype had produced callus that was regenerated into shoots in previous experiments (Han *et al.*, in review), and the stem internodes which were placed on non-selective medium in this experiment also produced callus (Table 1). When this experiment was repeated, except that the stems were cultured on selective medium with lower levels of hygromycin (15 ug/ml) and kanamycin (15 ug/ml), no resistant calli were observed.

The results of these experiments raised the possibility that the production of transgenic callus, in a manner similar to the *in vitro* responses of normal black locust tissues, could be strongly genotype-dependent. If this was the case, then particular genotypes may be recalcitrant to the production of transgenic callus under the conditions that were tested. Given the usual design of tissue culture experiments, in which a range of culture conditions/media components are tested on one species, seedlot, or genotype, it seemed appropriate to reverse the design. The question then became: can genotypes be identified which are superior for transgenic callus production through the use of this particular inoculation and culture protocol?

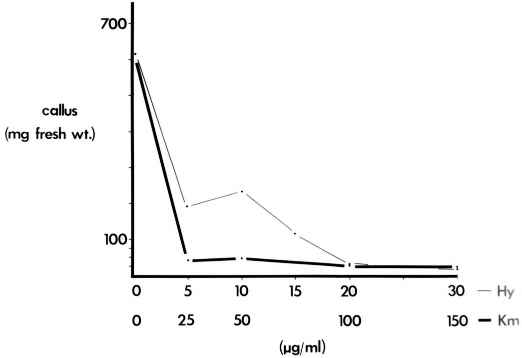

Figure 2. Inhibition of callus formation by stem segments of *Robinia pseudoacacia*, due to the presence of various levels of hygromycin or kanamycin in the culture medium.

Table 1. Number of explants screened for production of transgenic callus after inoculation of stem tissues obtained from tree #1 with *Agrobacterium*-based vectors.

Strain (treatment)	No. of explants[1]	Callus production	Average weight (g)
pGV3850HPT::pKU2	63	no	-
pGV3850HPT::pKU2 (+ AS)[2]	64	no	-
A136 (+ or - AS)	15	no	-
A281 (pCIB715)	40	no	-
pGV3850HPT::pKU2	12[3]	yes	1.076

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[1] The number of explants transferred to medium containing hygromycin (20 ug/ml) or kanamycin (100 ug/ml) was combined.

[2] Acetosyringone (to a concentration of 100 uM) was added to the bacteria 5 hours prior to inoculation of the explants.

[3] These explants were transferred to non-selective medium.

Seedling-derived stem tissues were selected as inoculation sites for these genotype screening experiments, since a reasonable number of clonal explants could be generated over a short period of time by propagating shoot cultures of individual seedlings. The disarmed cointegrate vector was used for inoculations, since it was not known if the expression of *onc* loci (which could be transferred by the binary strain) would affect the growth of black locust cells on the selective medium. A total of 845 stem explants from 67 individuals were inoculated with the cointegrate vector, and the results of the experiment are shown in Table 2. After 4 weeks of culture on selective medium, only 13 explants had calli that were still growing. Nine of these explants were obtained from a single seedling from tree #2; three were from a seedling in the random seedlot, and another was obtained from tree #5. All non-inoculated explants that were placed on non-selective medium proliferated into callus, and non-inoculated explants that were placed on selective media died. AOV of these data showed that differences due to family and antibiotic type were non-significant, whereas differences attributed to individual genotypes were highly significant (F-test, alpha < .01).

It was often observed that apparently resistant callus would proliferate from stem explants, but would then die after excision or transfer to fresh selective medium. This occurred to most of the tissues scored as "resistant" in

Table 2. Formation of calli after inoculation with pGV3850HPT::pKU2 on callus medium containing hygromycin (20 ug/ml) or kanamycin (100 ug/ml). Data were collected after four weeks of culture (one week subculture interval).

			No. calli / No. explants	
Source	Total no. clones	No. clones that produced callus	Hy^r	Km^r
Random	13	1	3/8	-
#1	13	0	-	-
#2	13	1	5/5	2/4
#3	14	0	-	-
#5	14	1	1/6	-

---

[1] #1, #2, #3, #5, and Random designate seeds collected from trees #1, #2, #3, #5, and from randomly selected trees, respectively.

this experiment, such that DNA could only be isolated from one kanamycin resistant callus produced by the tree #2 seedling (this genotype was designated 2.4). The rest of the calli died 2-3 weeks after data were collected. A substance that comigrated with authentic nopaline was detected in the kanamycin resistant tissue (Figure 3). Sequences related to the *npt*II probe were found in the DNA from the kanamycin resistant callus, but not in the normal callus DNA (Figure 4).

The stem inoculation experiment was repeated with stem segments from 2.4, and six other genotypes that were tested in the original experiment. After 3 weeks of culture on selective medium, only five explants showed evidence of callus formation. All were produced by genotype 2.4, on medium containing hygromycin. After data were collected, all the explants were transferred to, and maintained on, selective medium with lower levels of hygromycin (10 ug/ml) and kanamycin (50 ug/ml). This was done to increase the growth rate of the resistant calli, while still inhibiting proliferation of normal tissues. Still, no kanamycin resistant calli were detected in this experiment. As a result of this relaxation of selection, however, explants from other genotypes also began to proliferate callus on medium containing hygromycin (Table 3). This proliferation could have been due to a release of growth suppression of transgenic cells which were unable to divide in medium containing the higher level of hygromycin.

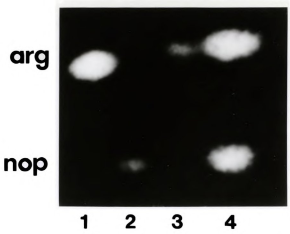

Figure 3. Paper electrophoretogram of ethanol-extracted kanamycin resistant callus incited on genotype 2.4 by *Agrobacterium* strain pGV3850HPT::pKU2. Lanes: 1) arginine; 2) kanamycin resistant callus; 3) normal callus; 4) nopaline and arginine.

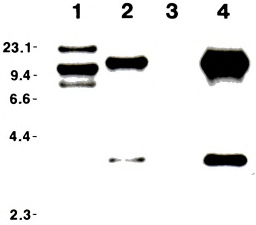

Figure 4. Autoradiograph of a Southern blot containing DNA from kanamycin resistant callus of black locust. Lanes: 1) kanamycin resistant callus (6 ug DNA); 2) pGV3850HPT::pKU2 (10 ng); 3) normal callus (6 ug); 4) pGV3850HPT::pKU2 (50 ng). The blot was hybridized to the *npt*II locus from pCIB10. Molecular size markers (kb) are along the left margin.

Nopaline was not detected in any of the putative transgenic calli from this experiment, however. One possible explanation for this result is that the expected T-DNA had not been transferred to these cells, so these calli were normal cells that had escaped the screening procedure. Alternatively, the T-DNA may have been present in these cells, but the nopaline was not detected due to low levels of expression, or due to interruption or absence of the locus (analogous to the results reported in Chapter 2 of this dissertation). To distinguish among these possibilities, DNA was isolated from all calli that were greater than 300 mg fresh weight. Seven of the lines yielded DNA sufficient for Southern analysis. Five to six ug of DNA from each callus was digested with *Hin*dIII or *Eco*RI, the fragments were separated in agarose gels, blotted to nitrocellulose, and *hpt*-specific probe DNA was hybridized to the blots. Although T-region fragments in 10 ng of bacterial DNA were readily detected on these autoradiographs, hybridization was not detected between the probe and putative transgenic callus DNA.

This result was unexpected, since the goal of this experiment was simply to repeat the earlier trial in which the transgenic (kanamycin resistant) callus was obtained. The same genotype that appeared desirable for transgenic callus proliferation in the original stem inoculation experiment, also appeared as such in the second experiment. However, the inability to detect T-DNA sequences in these

calli raises the possibility that the majority of the cells in the tissue may not have been transgenic. As such, the proportion of cells that contained T-DNA may have been zero, or sufficiently small so as to escape detection by Southern analysis. If no cells contained T-DNA, then one would predict that the stem tissues of certain genotypes, including 2.4, could proliferate naturally in medium containing hygromycin or kanamycin. This would occur, for example, if uptake of aminocyclitol antibiotics was reduced in some genotypes relative to others. Natural resistance to kanamycin or hygromycin was not observed, however, since the presence of either antibiotic in the culture medium inhibited callus growth in all the genotypes that were tested (Table 4).

An alternative possibility is that few transgenic cells were present at the medium/callus interface, and that these cells effectively phosphorylated the hygromycin prior to its acropetal diffusion into the tissue mass. The chimeric *hpt* construct in pGV3850HPT::pKU2 had a CaMV 35S promoter region, while the *npt*II construct had the 1' promoter from the TR-region of pTiA6 (Baker *et al.*, 1987). Messenger RNA levels were 8- to 15- fold higher with loci linked to the 35S promoter compared to the same loci linked to the 1' promoter region in two different plant species (Harpster *et al.*, 1988). If the CaMV35S-*hpt* construct results in high levels of *hpt* expression in black locust cells, then it is

Table 3. Average weights of explants after 10 weeks of culture on selective medium, following inoculation of explants with *Agrobacterium* strain pGV3850HPT::pKU2. The latter 8 weeks of culture was on medium with kanamycin (50 ug/ml) or hygromycin (10 ug/ml).

		Average weight (mg)	
Source	Clone	Kanamycin	Hygromycin
1	8	48[1]	352
2	4	85	2078
3	8	74	858
3	10	36	57
R	7	79	132
R	9	21	39
R	10	75	411

[1] Average of five explants per datum.

Table 4. Weights of uninoculated stem segments after 4 weeks of culture on callus induction medium containing no antibiotics, hygromycin, or kanamycin.

	Average weight (mg)			
Source	MSH	Hyg (10)	Hyg (20)	Kan (50)[1]
2.4	1265[2]	46	53	19
Range of five other genotypes	14 - 1751	17 - 116	14 - 28	1 - 13

[1] Media contained MS with 5 uM NAA and 5 uM BAP only (MSH), or supplemented with hygromycin (10 or 20 ug/ml) or kanamycin (50 ug/ml).

[2] Values are the average of at least 5 explants.

possible that relatively few transgenic cells with single copies of the *hpt* locus could efficiently detoxify the hygromycin in the callus, yet be undetectable after DNA isolation. This phenomenon could be avoided if transgenic black locust cells were maintained in selective medium while in suspension. This alternative strategy is plausible, since cells of several different black locust genotypes have been successfully maintained in suspension cultures (Han, unpublished data).

Agrobacterium pGV3850HPT::pKU2 was successfully used in these experiments to transfer antibiotic resistance loci into black locust cells. This vector is similar to *A. tumefaciens* strain A208, which produced crown galls on a relatively high proportion (30%) of the explants that were inoculated (Chapter 1, Table 1). This vector should prove useful in subsequent investigations designed to produce transgenic callus in black locust. However, modification of the T-region of a cointegrate strain for transfer of other DNA sequences of interest requires more effort than does the construction of a binary strain (Klee *et al.*, 1987). In view of the long-term goals of the research on black locust, it may prove useful to produce several different binary strains, which differ with respect to the helper strain, and test the ability of those strains to transform black locust cells. Particular bacterial strain/plant genotype combinations may be discovered which are particularly

efficient, and will increase the ease with which DNA sequences can be introduced into the genome.

In all of these experiments, the phenotypes of non-transgenic tissues cultured on medium which contained hygromycin differed dramatically from the phenotypes of normal tissues on kanamycin. Normal stem tissues cultured on medium with hygromycin turned dark brown, with the exception of the nodes, which remained green. The dark brown internodal tissues could have been due to the production of tannins or phenolic compounds which are often associated with cell death. In contrast, the putative transgenic callus tissue was white. Explants that were cultured in medium with kanamycin appeared white and often increased in diameter. This was also observed during culture on kanamycin after inoculation of explants with the *Agrobacterium*, making it difficult to distinguish transgenic cells from the normal tissues. The use of hygromycin as a selectable marker appeared to permit simpler visual selection of transgenic black locust cells as compared with kanamycin. It can not be determined, however, if either marker is functionally superior on the basis of these experiments.

It is encouraging to report that transgenic callus was successfully produced on tissues of *Robinia pseudoacacia* from both a binary vector, and from a disarmed cointegrate vector. Although it appears that certain genotypes may have a predilection for transgenic callus production, this could

not be proved in these experiments, since T-DNA sequences were not detected in the apparently hygromycin resistant calli observed in the second stem segment inoculation experiment. However, since several studies of other legumes have indicated that genetic factors play a significant role in determining *in vitro* responses, and the analysis of variance performed on the data in Table 2 revealed highly significant effects of genotype, it seems that a reasonable working hypothesis is that genetic factors have an effect on transgenic callus production in black locust.

It is of interest to speculate on the possible roles some of these genetic factors may play in this system. Certainly, the ability of a genotype to produce callus would be considered a necessary trait for the production of transgenic callus. The results from inoculation experiments performed on stem segments of mature tree #1 (Table 1), however, suggest that this is not sufficient for the production of transgenic callus in the presence of a selective agent. Some genotypes may not be transformable by *Agrobacterium*, possibly due to the low number of cells that are both competent to receive the T-DNA and can also undergo mitosis. It appeared that tree #1 could be transformed, since tumors were observed (albeit rarely; 1 out of 10 explants) after inoculation with *A. tumefaciens* strain A208 in previous experiments. If a number of closely spaced transformed cells are required to form a transgenic callus,

then perhaps only genotypes which are transformed more frequently can form such a callus. Many other factors could be important; the tissues of some genotypes may be particularly sensitive to wounding, the presence of bacteria in conductive tissues, the use of cephalosporin antibiotics that kill the *Agrobacterium*, or the effects of localized cellular death (and the subsequent leakage of vacuolar contents that accompanies it).

An analogy may be drawn between guidelines for the selection of genotypes from wild stands for use in an applied breeding program (Ledig, 1973), and the selection of genotype 2.4 for further use in gene transfer studies. Although wild stands of trees are genetically and environmentally heterogeneous, selections of straight stemmed, non-diseased individuals are usually made prior to rigorous progeny testing. Even though there is no proof that the selected individuals are genetically superior to other trees, they have at least exhibited phenotypic superiority in one particular environment, and are therefore worthy of further study (*i.e.* progeny or clonal testing). Similarly, it seems prudent to test genotype 2.4 in future experiments designed to produce transgenic callus, since this genotype has demonstrated phenotypic superiority in the inoculation and culture protocols that were employed in the experiments described in this chapter. Future studies should focus on the production of sufficient quantities of

transgenic callus to allow growth maintenance and regeneration studies.

Barriers certainly remain to the routine production of transgenic callus in *R. pseudoacacia*. With two exceptions (the kanamycin resistant lines analyzed in Figures 1 and 3), the putative transgenic tissue lines that were obtained in these experiments invariably turned brown after 4-8 weeks of culture. The kanamycin resistant lines, while subculturable, grew so slowly as to preclude meaningful experiments designed to induce organogenesis. Similar losses of tissue viability have also been observed with protoplast cultures of *R. pseudoacacia*, where growth and cellular division beyond the microcallus stage was not observed (Han and Keathley, 1987). In addition, shoot morphogenesis often occurs unexpectedly from calli derived from seedlings, while the tissues are growing on callus growth medium (Davis and Han, unpublished observations). These observations underscore the need for more basic information regarding the phytohormonal regimes necessary to maintain vigorous callus growth and induce organogenesis in black locust cells and tissues, such that these processes can be controlled more effectively.

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CONCLUSIONS AND RECOMMENDATIONS

Unselected populations of most forest trees are genetically highly heterogeneous (Adams, 1983; Zobel and Talbert, 1984). This provides abundant raw material for fledgling conventional tree improvement programs, such as the ones currently being developed for *R. pseudoacacia* (Kennedy, 1983; Mehbrahtu and Hanover, 1989). As shown by the results of the experiments described in this dissertation, this genetic variability also appears to have a major impact on the development and application of tissue culture techniques that are designed to micropropagate mature trees, or introduce foreign genes into somatic cells via *Agrobacterium*-mediated gene transfer.

The results of the experiments described in the first chapter indicate that mature (greater than 20 years of age) individuals of *Robinia pseudoacacia* can be successfully micropropagated by *in vitro* bud culture. It is feasible that one of the mature trees (#1, described in Chapter 1) could be successfully micropropagated in this manner on a large-scale, commercial basis. Bud culture is therefore an effective tool by which some mature black locust trees can be propagated, while imposing minimum damage to the donor tree. This should prove useful for cloning some genotypes for basic genetic and physiological research purposes.

The practical application of this technique to the breeding program must await a systematic sampling of the

breeding population in order to assess the efficacy of bud culture on these individuals. A series of experiments designed to measure the heritability of responsiveness to bud culture would help quantify the relative contribution of genetic factors and environmental factors such as site, to the variability observed in this character. Based on results obtained in other legumes (Bingham *et al.*, 1975; Reisch and Bingham, 1980), genetic factors will most likely be found to play an important role in determining individual tree responses to tissue culture. If this is the case with black locust, then these *in vitro* techniques can be successfully integrated into the tree improvement program if the tree-specific responses to these techniques are exploited in the early stages of the program. That is, *in vitro* performance should be used as a selection criterion for inclusion of a particular family or individual in the breeding pool.

These results were based on a small sample size of only five mature trees, and may not be predictive of the *in vitro* response that will be observed in a black locust breeding population. If a relatively large proportion of trees responds well to the culture regimes described in this study, then selection of good *in vitro* performers will not be a limiting factor in the selection scheme. If, on the other hand, it is desirable to select and clone specific genotypes that do not respond well to the culture regimes

that were tested in this study, these results do not preclude the development of modified culture regimes designed to successfully propagate an individual genotype or set of genotypes.

Perennial (age-dependent) patterns in expression of bud culturability should also be explored in *R. pseudoacacia*. If such developmental regulation can be predicted, then it may be possible to employ *in vitro* screening, allowing selection of superior performers at the seedling stage, prior to field planting.

The results of the experiments described in Chapter 2 demonstrate that T-DNA from the Ti plasmid of *Agrobacterium tumefaciens* is stably maintained in the genome of *R. pseudoacacia* when explants from seedlings are inoculated with the bacteria *in vitro*. It was important to demonstrate this fact, since no previous studies could be found in which susceptibility of black locust trees to crown gall tumor disease had been demonstrated. Results of a partial T-DNA mapping strategy suggested that the T-DNAs were truncated on the right ends in three of the tumor lines, but similar results have been observed in plant species known to be hosts for *A. tumefaciens*. Therefore, successful T-DNA transfer, integration, and expression were not expected to be barriers to the use of engineered *Agrobacterium* strains to introduce foreign genes encoding antibiotic resistance loci into the black locust genome.

The results reported in Chapter 3 indicated that callus can be induced to grow on selective media containing the aminoglycoside antibiotics kanamycin or hygromycin, both of which are toxic to normal cells of black locust, after inoculation of explants with modified *Agrobacterium* strains. However, the genotype of the donor plant had a significant effect on the ability of the transgenic cells to proliferate on medium which contained high levels of the antibiotic, and the only calli that were proven to be transgenic were selected on kanamycin. Even if the stem tissues of a large number of different individuals could be engineered in this fashion, the maintenance of callus growth, and obtaining plantlet regeneration remain as barriers to the application of this technique to the production of genetically engineered black locust trees. For basic research purposes, however, clonal tissues of the most responsive genotype that has been identified can be used to test various culture conditions. These experiments should be designed to increase the vigor and morphogenic potential of transgenic tissues. One possible strategy is to transfer putative transgenic calli to medium lacking the selective agent for further growth and regeneration.

It is of interest to note that the capacity of seedling tissues to proliferate into shoot cultures appears high and fairly uniform across individual seedlings, compared to the individual tree responses that are characteristic of mature trees. This phenomenon has also been observed in several

conifer species (*e.g.*, Mott and Amerson, 1984). The desirable seedling response is important in the context of the black locust breeding program, since it indicates that micropropagation should prove useful for increasing the number of propagules from genetically valuable seed (such as from controlled pollinations). The individual tree responses observed in mature trees are important if transgenic shoots are eventually regenerated from tissues of older trees. These shoots should be produced from individuals whose proliferation in shoot cultures, and in rooting medium, is acceptable. This will minimize the possibility that a lack of vigorous shoot proliferation and rooting will present a barrier to the production of transgenic plantlets.

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