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Agrobacterium tumefaciens-mediated transformation of several *Rubus* genotypes and recovery of transformed plants

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Abstract

Experiments in shoot regeneration and virulent *Agrobacterium tumefaciens*-mediated transformation were used to develop a protocol for *Rubus* transformation. This protocol was then used to produce transformed *Rubus* plants from *in vitro* internodes inoculated with an *Agrobacterium tumefaciens* encoding neomycin phosphotransferase on its disarmed T-DNA. Two transformed plants were selected from 800 inoculations on a medium containing 10 μg ml^{-1} kanamycin. Results indicated that this level of kanamycin successfully selected against non-transformed cells but did not reduce the number of transformed, kanamycin-resistant, shoots formed. Enzyme assays and Southern blot analysis verified the presence of the β-glucuronidase gene in the plant genome.

Abbreviations: BA – benzyladenine, CAT – chloramphenicol acetyl transferase, CSR – chlorsulfuron resistance, 2,4-D – 2,4-dichlorophenoxyacetic acid, β-GUS – β-glucuronidase, IAA – indoleacetic acid, IBA – indolebutyric acid, NAA – naphthaleneacetic acid, NPT – neomycin phosphotransferase, TDZ – thidiazuron

Introduction

Several genes have been introduced into many herbaceous plant genera using *Agrobacterium tumefaciens*-mediated transformation, but this technique has worked with relatively few woody or perennial species (e.g. Colby et al. 1991; Fillatti et al. 1987; James et al. 1989, 1990; Nehra et al. 1990; Smigocki & Hammerschlag 1991). Even when genes have been inserted into woody species, the number of transformed shoots obtained has been relatively small compared to the number of transformation attempts. Several factors have slowed adaptation of transformation techniques to woody plants: regeneration protocols are still being developed for many woody plants; the biology of the *Agrobacterium tumefaciens*/host plant interaction appears to be more complicated in woody species; and woody plants often show unusual sensitivity to kanamycin, which prevents the use of the standard kanamycin resistance selective marker in *Agrobacterium tumefaciens*-mediated transformation (Colby & Meredith 1990; Fiola et al. 1990; James et al. 1989; Matthews & Litz 1990).

Therefore, experiments were conducted to address these issues involved in *Agrobacterium tumefaciens*-directed transformation of *Rubus*. Data from initial experiments were used to develop a protocol for transformation of *in vitro*

clonal *Rubus* and regeneration of transformed plantlets. Plants recovered from this method were used for marker enzyme assays and Southern analysis to verify transformation.

Materials and methods

Throughout these experiments, *in vitro* explants (originally derived from excised meristems of greenhouse-grown plants) were grown in continuous light on pH 5.7 medium containing MS (Murashige & Skoog 1962) inorganic salts with 55.5 μM myo-inositol, 0.3 mM adenine sulfate, 87.2 mM sucrose, 2–5 μM BA and 0.7% Sigma agar (Fiola et al. 1990). For cotyledons, ripe fruit from controlled crosses in a greenhouse were surface disinfested in 0.52% sodium hypochlorite for 10 min and the seeds were excised from their pyrenes under a dissecting microscope (Fiola et al. 1990).

Plant regeneration experiments

Experiments were conducted to determine the effects of several biochemicals on the ability of 'Dirksen Thornless' and MD-ETCE-1 ('Black Satin' × 'Tayberry') clonal *Rubus* leaf and internode segments to regenerate shoots. Chemicals were added to a MS-based organogenesis medium as previously published but containing only the plant growth regulators indicated below (Swartz et al. 1990). Leaf regeneration techniques were as previously published (Fiola et al. 1990). The internode sections were approximately 0.5 to 1 cm long as excised 3 mm above and below adjacent nodes. These pieces were placed on their side on the medium. Twenty 30-ml glass bottles containing 10 ml of media were used per treatment. Four excised leaves or internode sections were cultured per bottle and each experiment was performed twice. The chemicals investigated were:

1. Cytokinin Experiment – 0, 5 or 15 μM of each of two cytokinins, BA and TDZ (no auxins added);
2. Auxin Experiment – 0, 0.5, 2.5 or 5 μM of each of four auxins, IAA, IBA, NAA and 2,4-D (5 μM TDZ added to all cultures); and
3. Antibiotic Experiment – 0 to 250 $\mu g\ ml^{-1}$ kanamycin and 0 to 1 mM cefotaxime, the antibiotics used to repress the growth of untransformed tissue and reduce *A. tumefaciens* populations, respectively (5 μM TDZ added to all cultures).

In separate treatments in Experiment 3, MD-ETCE-1 internodes were also incubated with or without disarmed *Agrobacterium tumefaciens* strain GV 3101 and placed on medium with 10 $\mu g\ ml^{-1}$ kanamycin and 0.4 mM cefotaxime.

Transformation experiments

In Experiments 4 to 6, the bacterial inoculum was prepared by overnight incubation at 28 °C in liquid LB medium (Maniatis et al. 1982). Individual shoots were inoculated at three internodes using a sterile fine-drawn glass Pasteur pipette to inject μl quantities of the inoculum into the wound site. Leaves were wounded by an excision cut across petioles. Cotyledons were wounded by making a single transverse cut to separate the distal two-thirds of the cotyledons from the embryo axis, which was discarded. These tissues were immediately immersed in the bacterial inoculum for 10 min (leaves) or 60 min (cotyledons). After coincubation on Whatman #3 filter papers saturated with liquid MS medium without growth regulators for 4 days (or for a period as noted in Experiment 5), explants were dipped for 30 sec in 0.48 mM carbenicillin to inhibit the *A. tumefaciens*. Twenty cultures were inoculated per treatment. Transformation efficiency was determined as the percentage of explants forming galls and the number of galls per explant after 2 months growth on MS medium with no growth regulators and 0.24 mM of carbenicillin.

In Experiment 4, the susceptibility of three different plant tissues to four gall-causing *A. tumefaciens* strains (C58, A6, 516 and 527) was investigated. MD-GCG-1 ('Cherokee' × 'VSPB-1') blackberry was used for leaf and internode inoculations and MD-GBZ ('Austin Thornless' × 'Tayberry') seeds were used for the cotyledon experiment. A plasmid of one of the virulent strains, pTiC58, has a *vir* region similar to

pGV3850, the disarmed plasmid used for transformation (Jefferson 1987). Strain C58 was originally isolated from *Prunus*, as was strain 527 (Ditta et al. 1980). Strain 516 was originally isolated from *Rubus*.

In Experiment 5, the coincubation period was varied after *A. tumefaciens* strains 516 or C58 were injected into the *in vitro* internodes of MD-GCG-1 blackberry. Coincubation was terminated after 1, 2, 4, 6 or 8 days by soaking the explants in 0.48 mM carbenicillin for 1 min.

The effect of phenolic substances on the virulence of *A. tumefaciens* was investigated in Experiment 6. MD-GCG-1 *in vitro* explants were inoculated by internode injection with strain 516 grown overnight in LB liquid medium with either 20 μM acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) or a combination of seven phenolic substances each at 45 μM: catechol, gallic acid, pyrogallic acid, *p*-hydroxybenzoic acid, protocatechnic acid, β-resorcylic acid and vanillin (Bolton et al. 1986).

Transformation in Experiments 4–6 was confirmed on a random sample of 10 galls from each treatment by paper electrophoretic separation and fluorescence visualization of opines (Otten & Schilperoort 1978). Each experiment was repeated.

All of the above experiments (1–6) were designed as randomized complete blocks and data were analyzed by ANOVA with means separated by LSD ($p = 0.05$). Data from percentage "explants regenerating" or "with galls" experiments were arcsin transformed before analysis by ANOVA; however, only non-transformed means are presented. Only consistently significantly different means, i.e. a $p < 0.05$ in both replications, are reported in the text unless otherwise noted.

Production of transformed plants

The above model studies were used to develop the following protocol for production of *Agrobacterium tumefaciens*-transformed *Rubus* plants. *In vitro* leaves, internode pieces or cotyledons were used as source plant material. Overnight cultures of *A. tumefaciens* C58 with disarmed plasmid pGV 3850 and T-DNA from mating with pJIT 106 or pJIT 54 were grown on LB liquid medium with 20 μM acetosyringone, 3 μM rifampicin and 50 μg ml^{-1} kanamycin (Jefferson 1987). The plasmid pJIT 106 contains genes for neomycin phosphotransferase (NPTII), β-glucuronidase (β-GUS) and chlorsulfuron herbicide resistance (CSR) under constitutive promoters (Fig. 1). The plasmid pJIT 54 contains genes for chloramphenicol acetyl transferase (CAT) and neomycin phosphotransferase (NPTII), also under constitutive promoters. The bacteria were pelleted at 10,000 × *g* for 10 min and resuspended in liquid MS without plant growth regulators or antibiotics. Leaves were excised by a single cut across the petiole, internodes were excised by cuts 3 mm above and below adjacent nodes, and cotyledons were separated from the embryo axis by a single cut across the cotyledons. These tissues were placed in the bacterial suspension for 10 min (60 min for cotyledons). The inoculated plant material was

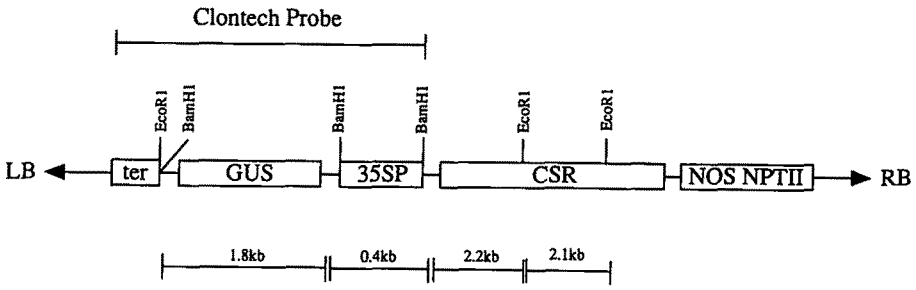

Fig. 1. Restriction map of the pJIT106 plasmid containing the β-GUS, CSR and NPTII genes and their promoters used to transform MD-ETCE-1 plants. The homologous region of the Clontech β-GUS probe used in the Southern hybridization blots is also indicated.

coincubated inside sealed jars containing Whatman #3 paper discs soaked with liquid MS medium (devoid of plant growth regulators). After 4 days in the dark, the explants were rinsed in 0.5 mM cefotaxime for 30 sec and placed on MS-based organogenesis medium containing 5 μM TDZ (Fiola et al. 1990), 10 μg ml^{-1} kanamycin, and 0.4 mM cefotaxime. Regenerated shoots were transferred for several subcultures on MS-based medium that contained 1 μM IBA, 15 μg ml^{-1} kanamycin and 0.4 mM cefotaxime. Rooted explants were assayed for β-GUS or CAT activity. The protocol for these assays was as previously published (Jefferson 1987; Davies et al. 1986).

β-GUS positive plants were used for Southern blot analysis. DNA was isolated from *in vitro* leaves after explants were grown in the dark for 2 days to reduce starch. Samples were collected from bacteria-free plants only, as determined by streaking tissue on LB and RS selective media (Burr et al. 1987). After leaf collection, nuclei were extracted in a hexylene glycol-based buffer and purified in a discontinuous percol gradient. The 60% percol fraction was lysed with Triton X-100 and centrifuged at 100,000 × *g* for 24 h in ethidium bromide-CsCl (Watson & Thompson 1988). The single band of plant nuclear DNA was collected and digested with BamH1 or EcoR1 followed by electrophoresis on 0.8% agarose gels (7 μg/lane) (Maniatis et al. 1982). A ^{32}P-labeled HindIII fragment containing the CaMV 35S-GUS-NOS poly A sites (Clontech; Palo Alto, CA; Cat No. 6019-2) was used to probe the Southern blots (Fig. 1).

Results

Organogenesis

A significantly greater ($p < 0.01$) percentage of MD-ETCE-1 internodes and leaves produced shoots when TDZ was used instead of BA (Table 1). However, this effect was insignificant for 'Dirksen' (Table 1). Overall, internodes and leaves were equally organogenic. No cytokinin type or concentration effects were observed for number of shoots regenerated per leaf or internode (data not shown).

Auxins did not consistently improve the percentage regeneration of internodes (data not shown). At concentrations > 2.5 μM, all auxins except IAA significantly decreased the percentage regeneration. Auxins had little effect on the number of shoots formed per internode except at 0.5 μM IBA (7.0 vs. 3.9 control). Auxins were subsequently not used in transformation experiments.

Kanamycin significantly reduced the growth of all explants (Table 2). Organogenesis was also reduced (0 μg ml^{-1} kanamycin = 45% regeneration; 10 μg ml^{-1} = 17%; 20 μg ml^{-1} = 2%). No organogenesis was obtained at higher concentrations. On those internodes that regenerated shoots, kanamycin reduced the number of shoots that formed (Table 2). In contrast, cefotaxime increased the number of shoots formed per regenerating internode (Table 2); however, its slightly stimulatory effect on growth and percentage organogenesis was not consistently significant over the two replications of the experiment.

Table 1. The effects of benzyladenine (BA) and thidiazuron (TDZ) on the percentage regeneration from MD-ETCE-1 and 'Dirksen' internodes and leaves. Replicate experiment data were combined, the no cytokinin control data were not included in the ANOVA.[a]

	Internodes		Leaves	
	MD-ETCE-1	Dirksen	MD-ETCE-1	Dirksen
Control	0	0	0	0
5 μM TDZ	99	36	96	50
15 μM TDZ	95	8	81	52
5 μM BA	48	31	27	18
15 μM BA	31	16	15	13

[a] For MD-ETCE-1, TDZ was significantly better ($p < 0.01$) than BA for both internodes and leaves; for Dirksen, no differences were significant.

Table 2. The effects of kanamycin and cefotaxime on the fresh weight (mg) and number of shoots formed per regenerating internode in both replicate experiments (Rep1/Rep2) (genotypes combined).

Kanamycin concentration ($\mu g\ ml^{-1}$)	Shoots formed	Fresh weight
	Rep1/Rep2	Rep1/Rep2
0	2.6/3.3	596/804
10	1.6/2.4	331/184
20	0.0/0.5	31/43
30	0.0/0.0	19/20
50	0.0/0.0	13/13
100	0.0/0.0	7/9
LSD (5%)	0.4/0.4	122/94
Cefotaxime concentration (mM)		
0	3.7/3.3	309/528
0.2	5.6/4.6	424/352
0.4	4.0/5.7	431/448
0.6	4.8/4.5	466/418
0.8	4.7/4.6	642/578
1.0	5.8/5.3	692/487
LSD (5%)	0.2/1.0	118/157

When 10 $\mu g\ ml^{-1}$ kanamycin and 0.4 mM cefotaxime were combined in the medium, the percentage organogenesis was significantly reduced (control = 85%; kanamycin + cefotaxime = 18%). The presence of *A. tumefaciens* in the coincubation medium had no significant effect on organogenesis (kanamycin + cefotaxime + disarmed *A. tumefaciens* = 8% regeneration).

Gall formation

Opines were always detected in more than 80% of the galls examined throughout these studies. *Agrobacterium tumefaciens* strains differed in their ability to cause gall formation (Table 3). Strain 516, isolated from *Rubus*, was more virulent on all plant parts ($p < 0.01$). Cotyledons and internodes were as susceptible to gall formation as leaves.

In Experiment 5, four days of coincubation was optimal for transformation with both oncogenic strains (Fig. 2). The statistically significant ($p < 0.05$) quadratic line of best fit

Table 3. The effect of *Agrobacterium tumefaciens* strain type and plant tissue on the percentage of successful gall formation. Data from both replicate experiments were combined.[a]

Strain	Leaves	Internodes	Cotyledons
A6	11	13	55
C58	18	32	48
516	51	69	75
527	17	16	20

[a] Strain 516 was significantly better ($p < 0.01$) than other strains for each explant type; no other differences were significant.

was: $Y\ (\%\ \text{galls}) = 2.8\% + 23.6 \times (\text{days}) - 2.4\ X^2$ (days).

Acetosyringone, and to a lesser extent the seven combined phenolic compounds, significantly ($p < 0.05$) improved the number of galls formed per shoot compared to the control (control = 1.6 galls/shoot with 3 inoculations; acetosyringone = 2.3 galls/shoot; combined phenolics = 1.9 galls/shoot).

Plant transformation

Approximately 50 shoots regenerated after coincubation of various *Rubus* explants with disarmed *A. tumefaciens* strains containing NPTII/β-GUS and NPTII/CAT and subsequent growth on organogenesis medium containing 10 $\mu g\ ml^{-1}$ of kanamycin. After several month-long subcultures, only two β-GUS-putative and five CAT-putative transformants did not become chlorotic or necrotic (Table 4). These rooted on medium containing 15 $\mu g\ ml^{-1}$ kanamycin. All of these exhibited enzyme activity indicating transformation (Figs 3, 4). Some callus formed from non-regenerating inoculated explants, but this tissue became chlorotic and did not exhibit marker enzyme activity.

The quantities of DNA isolated from MD-ETCE-1 control plants (26 $\mu g\ g^{-1}$ fresh weight of tissue) and β-GUS-transformed plants (14 $\mu g\ g^{-1}$ fresh weight of tissue) contained only small amounts of RNA and were readily digested by restriction endonucleases. BamH1 digestion of DNA from two transformed explants yielded several fragments that hybridized with the β-GUS probe (Fig. 5). One fragment matched the size of the β-GUS gene (about 1.8 kbp) in

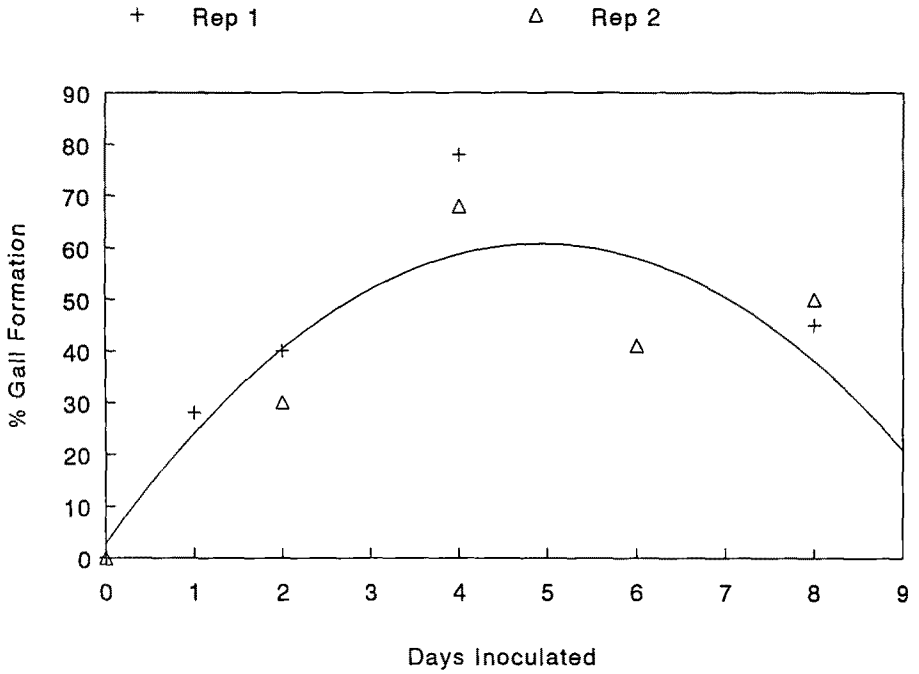

Fig. 2. The regression of length of coincubation time on percentage gall formation on MD-GCG-1 internodes inoculated with *Agrobacterium tumefaciens* strains 516 or C58 (replications combined). ($p < 0.05$; $r^2 = 0.80$)

Table 4. Number of *Agrobacterium*-inoculated explants regenerating shoots (that eventually rooted) from *Rubus* cotyledons, clonal stem internodes and leaves on medium containing 10 μg ml^{-1} kanamycin, 0.4 mM cefotaxime and 5 μM TDZ. All rooted explants had positive enzyme assays.

Genotype	Explant source	Bacterial plasmid (marker gene)	Number of explants inoculated	Number of regenerated shoots that rooted
MD-ETCE-1	Leaf	pJIT54 (CAT)	600	2
MD-ETCE-1	Internodes	pJIT106 (β-GUS)	800	2
MD-IAS[1]	Cotyledons	pJIT54 (CAT)	200	3

[1] MD-IAS are seedlings from 'Dirksen' × 'Shawnee' crosses.

the T-DNA of the plasmid pJIT106 (Fig. 1), indicating successful transfer of T-DNA from *Agrobacterium tumefaciens* into the plant genome. Digestion with EcoR1 also yielded several fragments, one of which was 4.4 kbp. This matched the DNA fragment between 2 EcoR1 sites in pJIT 106 consisting of 1.8 kbp of β-GUS gene plus 0.4 kbp of CaMV 35S promoter and a 2.2 kbp fragment from the CSR gene. An additional band was found near 2.2 kbp when transformed plant and pJIT 106 plasmid samples were digested with EcoR1, which may indicate the presence of an unknown restriction site. In one of the two β-GUS-transformed plants, several large fragments also hybridized with the probe (Fig. 5). The other transformant had hybridizing fragments similar to the plasmid and an additonal larger weight fragment.

Discussion

In a previous report on an effort to transform *Rubus*, no attempt was made to verify the

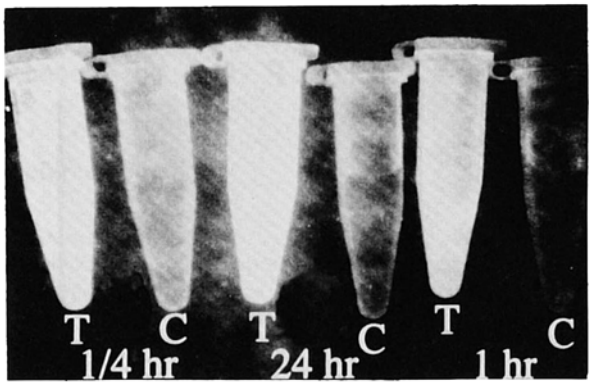

Fig. 3. β-GUS assay showing strong fluorescence from transformed leaf tissues of MD-ETCE-1 plantlets (labeled T). The assay reaction was terminated by Na_2CO_3 after 1/4 h, 24 h and 1 h (left to right) respectively. No fluorescence was detected from leaves of non-transformed plantlets (labeled C) at all tested reaction times.

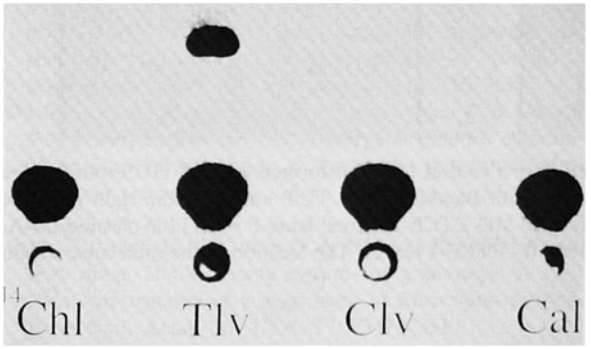

Fig. 4. Chloramphenicol acetyltransferase (CAT) assay in MD-ETCE-1 tissues. Lane ^{14}Chl shows ^{14}C-labeled chloramphenicol incubated without the addition of tissue extract. Lane Tlv shows CAT expression in a transformed leaf extract. No CAT activity was observed in lane Clv, non-transformed (control) leaf extract, or lane Cal, callus isolated from inoculated leaves. The reaction products were analyzed by thin layer chromatography, which separated the acetylated products (top) from the non-acetylated chloramphenicol (large spot) and the origin at the bottom (Davies et al. 1986).

T-DNA segment within the plant genome (Graham et al. 1990). The dot blot hybridization presented could have been a false positive from endophytotic *Agrobacterium* DNA. There was no evidence that plasmid DNA was eliminated in their DNA isolation procedure and *Agrobacterium* are sometimes difficult to isolate from plant tissues and can be present even though none were detected on nutrient agar (Burr et al. 1987). In the study presented here, transformation of MD-ETCE-1 plantlets with T-DNA containing β-GUS, NPTII and CSR genes was confirmed using Southern blot analysis. On one transformant, the presence of several bands larger than that from the plasmid pJIT 106 in Fig. 5 may indicate multiple integration into the plant genome.

Although transformation was achieved, the rate of transformed plant formation was very low as was found in other woody species. In grape, which has been difficult to transform, the sites of transformation and shoot regeneration are different and necrotic areas often develop around wound areas (Colby et al. 1991). In addition, an *Agrobacterium* strain not commonly used in transformation, *A. vitis* (= biovar 3), is preferentially found infecting grape. These problems did not occur here in *Rubus*, nor were any parts of *Rubus* explants significantly less susceptible to *Agrobacterium tumefaciens*-caused gall formation (Table 3). The disarmed *Agrobacterium tumefaciens* strain used in this study was not isolated from *Rubus*; however, its oncogenic progenitor (strain C58) was virulent on *Rubus* (Table 3). An *Agrobacterium tumefaciens* strain isolated from *Rubus* produced the largest percentage of transformed plants when virulent strains were compared and a vector derived from this strain may be more effective in *Rubus*.

It has been suggested that the extreme sensitivity of some species to kanamycin may reduce the regeneration of transformed shoots when this antibiotic is used (Colby & Meredith 1990; Fiola et al. 1990; James et al. 1989; Matthews & Litz 1990). The following analysis based on our experiments suggest that, at 10 μg ml^{-1}, this is not true in *Rubus*. If all cells on and immediately below the cut surface of *Rubus* petioles are equally capable of undergoing transformation and organogenesis, an estimate of the probability of recovery of transformed shoots can be made. Scanning electron microscopy shows an average of 440 cells in the regeneration area of *Rubus* petioles. In addition, 20% of the leaves produced galls (i.e. have at least one transformed cell) after inoculation with tumorigenic C58 *Agrobacterium tumefaciens*. This suggests that one in 2200 cells (= potential shoots) would be both transformed and could take part in regeneration. In a 600 leaf shoot regeneration experi-

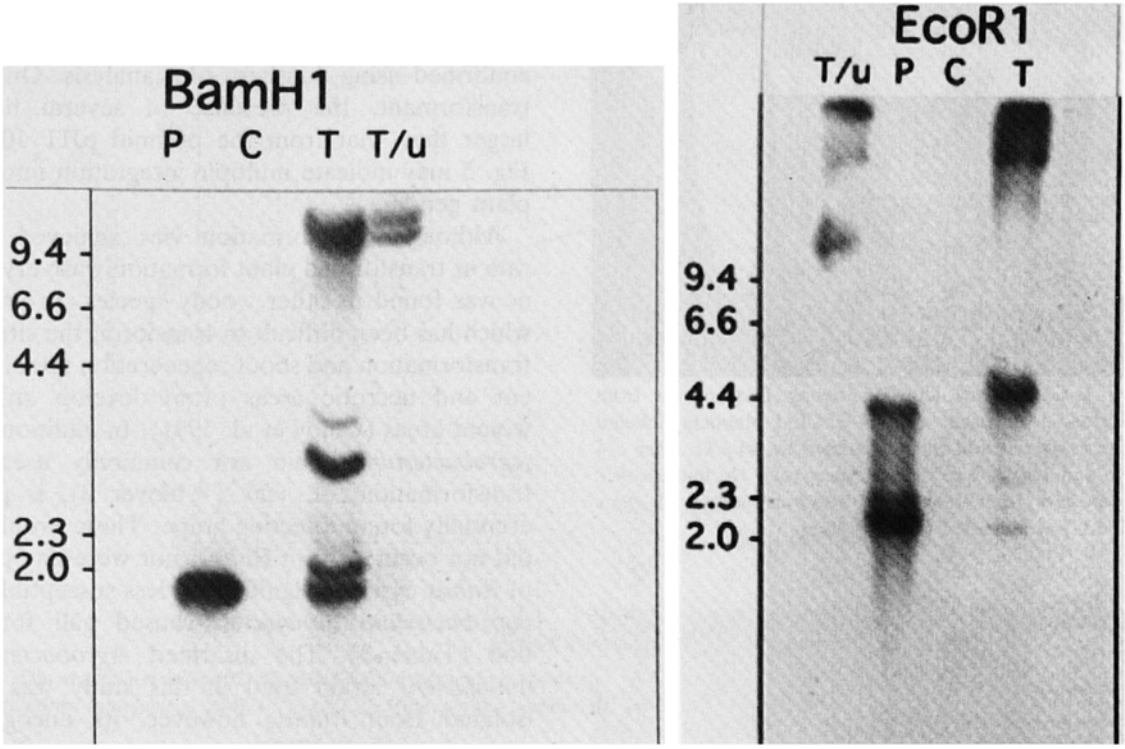

Fig. 5. Southern blot hybridization analysis of nuclear DNA isolated from a control non-transformed and a β-GUS transformed MD-ETCE-1 plantlet. Lanes C, P and T of the left gel were digested with BamH1. Lanes C, P and T of the right gel were digested with EcoR1. On each gel: T/u lanes were undigested transformed MD-ETCE-1 leaves; lanes P = pJIT106 plasmid DNA; lanes C = control MD-ETCE-1 leaves; lanes T = pJIT 106 transformed MD-ETCE-1 leaves. The location of the molecular weight markers are indicated on the left.

ment (as in Table 4), at least 2400 shoots should be formed on medium without kanamycin, since 4 to 5 shoots are formed per leaf petiole on 5 μM TDZ, 0 kanamycin and 0.4 mM cefotaxime containing medium (Table 2). If these assumptions are accurate, without selection, only one or two transformed shoots should be obtained from 600 leaf inoculations, similar to what was obtained (Table 4). Therefore, the use of 10 μg ml^{-1} kanamycin does not appear to be primarily responsible for poor rates of transformed shoot regeneration. In *Rubus*, inhibition of growth and rooting by kanamycin effectively eliminates a great proportion (> 98%) of presumably non-transformed shoots without reducing the theoretical yield of transformants.

In these *Rubus* genotypes, TDZ has been used to produce high rates of regeneration, auxins are not necessary and cefotaxime generally enhances regeneration (Tables 1 and 2; Fiola et al. 1990; Swartz et al. 1990). Since the regeneration protocol of MD-ETCE-1 has been optimized, increasing the number of transformed cells per inoculation should prove useful for increasing transformation efficiency. Four-day coincubation (Fig. 2) and the use of acetosyringone improved gall formation even when used with an *Agrobacterium tumefaciens* strain (516) isolated from *Rubus*. Many transformation efforts use 2-day coincubations and a previous attempt at *Rubus* transformation used 1-day (Graham et al. 1990). Cytokinins and auxins were not used in the coincubation medium for the experiments described here. However, in a more recent experiment, 25% more of the virulent *Agrobacterium*-inoculated explants produced galls when 5 μM BA and 1 μM IBA were added to the coincubation medium as compared to coincubation on medium without plant growth regulators. Cytokinins and auxins are produced by *Agrobac-*

terium cells and seem to be part of the induction physiology necessary for transformation (Alt-Moerbe et al. 1988).

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