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A risk assessment study of plant genetic transformation using *Agrobacterium* and implications for analysis of transgenic plants

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Abstract

Agrobacterium transformation systems for *Brassica*, *Solanum* and *Rubus*, using carbenicillin, cefotaxime and ticaracillin respectively to eliminate contamination, were examined for the presence of residual *Agrobacterium*. The results indicated that none of the antibiotics in question, succeeded in eliminating *Agrobacterium* and the contamination levels increased in explants from 12 to 16 weeks to such an extent that *Solanum* cultures senesced and died. This may be due to the fact that four times the Minimum bactericidal concentration values (concentration to be used for elimination of contaminants in culture), for the three antibiotics, were higher than the concentrations employed in the culture medium. Contamination in shoot material decreased over 16 to 24 weeks possibly due to bacteriostatis and the use only of the apical node for further culture. The presence of the binary vector was also noted under non-selective conditions, even up to 6 months after transformation, where approx. 50% of contaminated material still harboured bacterial cells with the binary vector at levels of approx. 10^7 Colony forming units per gram.

Abbreviations: CFU – colony forming units, GUS – β-glucoronidase, PCR – polymerase chain reaction, MBC – minimum bactericidal concentration, NPTII – neomycin phosphotransferase, RAPD's – random amplified polymorphic DNA

Introduction

With the discovery, in the early 1970's, of genetic transfer between *Agrobacterium* and the plant kingdom (Grierson and Covey, 1984), genetic engineers have now been able to use *Agrobacterium* as a vector for insertion of desirable genes into the plant genome. The origin of these genes may vary from unrelated plant species to microbes and animals (Dale et al., 1993), therefore, leading to many advances in genetic engineering. Transformation systems are now available for a large number of plant species and examples of transgenes for pest, viral, fungal, and herbicide resistance, and environmental stress tolerance exist (Dale et al., loc. cit.).

With these developments however, comes the problem of gene escape. The fear that genes inserted in plants will spread to wild plant populations or in the case of bacteria, spread giving them an advantage or disadvantage in their natural environment has now become a real issue. Such horizontal gene transfer is a well documented fact and has been reported not only to occur within species (Lorenz and Wackernagel, 1991; Lilley et al., 1994), but also between Gram positive and Gram negative bacteria (Schafer et al., 1990; Bertram et al., 1991). Heinemann and Sprague (1989) also give an example of bacterial plasmid transfer between bacteria and yeast.

When considering the use of *Agrobacterium* as a tool in genetic engineering, it is now imperative to also address the magnitude of risk posed to the environment in releasing transgenic plants. Transformation of plants is carried out by inoculation of explants with an *Agrobacterium* culture, co-cultivation for a

short period, followed by elimination of the bacterium with antibiotics (Draper et al., 1988). If, however, all the bacteria are not eliminated, then release of these plants may also result in release of the *Agrobacterium*. Various antibiotics have been employed to eliminate *Agrobacterium* following transformation, but very few authors actually test to ensure that the antibiotics succeed. Several examples of the use of carbenicillin at 500 mg l^{-1} (Radke et al., 1988; Moloney et al., 1989; da Camara Machado et al., 1992; Eapen and George, 1994) and more recently cefotaxime at 200–500 mg l^{-1} (Higgins et al., 1992; Hassan et al., 1993; Jacq et al., 1993; Calfalonieri, 1994; Howe et al., 1994; van der Hoeven et al., 1994) exist, but not one tested to ensure that bacterial elimination occurred.

There are few examples in the literature where sufficient screening for residual *Agrobacterium* was carried out. Graham and McNicol (1990) crushed plant material and plated it onto nutrient rich medium. Sarria et al. (1994) incubated plant material in liquid LB (Luria Broth) for seven days, and Gonsalves et al. (1994) plated strips of material onto LB medium. Two studies transferred material onto non - antibiotic medium, and when no *Agrobacterium* grew from the explants, they were presumed to be axenic (Barghchi et al., 1994; Mylnarova et al., 1994). This technique, however, is unreliable since bacteria can remain latent within plant tissue throughout the culture period (Cassells, 1991; Leifert et al., 1991). Antibiotics have been successful in the elimination of bacteria in tissue culture in some cases (Horsch and King, 1983; Leifert et al., 1991; Barrett and Cassells, 1994), but a vital aspect to their success is to ensure the completion of drug sensitivity tests, MBC determination, stability of the antibiotic and phytotoxicity to the plant.

This study was therefore carried out to investigate the ability of antibiotics to eliminate *Agrobacterium tumefaciens* after transformation using three model systems. With the persistence of residual bacteria, an examination to see if the binary vector was retained under non-selective conditions was carried out.

Materials and methods

Plant material

Seeds of rapid-cycling *Brassica oleraceae* were surface sterilized in 80% ethanol for one minute, 10% Domestos blech (Lever Bros, UK) for fifteen minutes, followed by two washes in sterile distilled water. These were germinated *in vitro* in the dark at 25 °C on Murashige and Skoog medium (Flow Lab, Irvine, UK) (Murashige and Skoog, 1962) with 20 g l^{-1} sucrose solidified with 8 g l^{-1} of agar (A - 7002; Sigma Chemical Company, Poole, Dorset, UK), and multiplied by nodal culture (Table 1). Cultures of *Solanum tuberosum* cv. 'Pentland squire' and *Rubus fruiticosus* L. agg cv. 'Loch ness', were also multiplied by nodal culture (Table 1). All material was subcultured at 6–8 week intervals and maintained at 25 °C under a 16-h photoperiod, light intensity 50 μmol m^{-2} s^{-1}, and 8-h night regime.

Agrobacterium transformation

The strain LBA4404 with the binary vector PBI121 (Jefferson, 1987a, b) containing the GUS gene and the NPTII gene, which confers kanamycin resistance, was employed for all transformation procedures. A single colony of LBA4404 was inoculated into LB broth containing 50 mg l^{-1} kanamycin (Sigma Chemical Co., Poole, Dorset, U.K.) and incubated overnight at 27 °C to serve as inoculum. Prior to transformation the bacterial cells were centrifuged, washed and finally resuspended in the appropriate tissue culture medium without kanamycin. This eliminated traces of the antibiotic which may have damaged the explants. Leaf discs 0.5 cm in diameter were cut from *Solanum* and *Rubus*, while internode segments of 0.3–0.5 cm in length served as explants from *Brassica* and *Rubus* cultures. Between 5 and 7 plates, with 10–15 explants/plate of each, were set up. Explants were transformed by using already published systems: *Brassica* - Millam, 1989; *Solanum* - Higgins et al., 1992; Hulme et al., 1992; *Rubus* - Graham and McNicol, 1990; Graham pers. comm. The antibiotics used to eliminate *Agrobacterium* are given in Table 2. Explants were subcultured on fresh medium every 10 days, and kanamycin selection was employed for only *Solanum* at this stage. *Brassica* and *Solanum* explants were transformed with an inoculum of LBA4404 of 3.7×10^7 cfu ml^{-1}. *Rubus* explants were transformed with a concentration of 2.9×10^7 cfu ml^{-1}.

Method of sampling for the presence of Agrobacterium

All surviving explants, which varied between 30-50 for each species, were sampled. Explants were cut longitudinally, weighed, and one half used for sampling, while the remaining half was placed on fresh antibiotic

Table 1. Nodal medium for *Brassica*, *Solanum* and *Rubus* (pH 5.6).

Component	*Brassica*		*Solanum*		*Rubus*	
	($g\ l^{-1}$)	(mgl^{-1})	(gl^{-1})	(mgl^{-1})	(gl^{-1})	(mgl^{-1})
MS basal salts	4.71	-	4.71	-	4.71	-
Sucrose	20.00	-	30.00	-	30.00	-
Calcium panthothenate	-	-	-	-	-	1.00
Cystene HCl	-	-	-	-	-	1.00
Glutamine	-	-	-	-	0.20	-
Glycine	-	-	-	0.40	-	2.00
Inositol	-	-	-	-	0.10	-
Nicotinic acid	-	-	-	0.10	-	1.00
Pyridoxine HCl	-	-	-	0.10	-	1.00
Thiamine HCl	-	-	-	0.91	-	1.00
Biotin	-	-	-	-	-	0.01
KH_2PO_4	-	-	0.17	-	-	-
$CaCl_2 \cdot H_2O$	-	-	0.44	-	-	-
GA_3	-	-	-	-	-	2.50
Agar	8.00	-	8.00	-	8.00	-

medium for *Agrobacterium* elimination. The explants were sampled in this manner because *Agrobacterium* travels in the xylem vessels (Tarbah and Goodman, 1987; Jones and Raju, 1988) and cutting longitudinally implies cutting though the transpiration system. Shoots were weighed following removal of the apical node, which was also placed onto fresh antibiotic medium. The explant or shoot was crushed with a small pestle in a 1.5 ml microcentrifuge tube of saline (0.85%), and placed on a shaker for 15 minutes. Following vortexing for 10 seconds, the samples were diluted serially and plated onto LB to determine which bacterial contaminants were present. Plates were incubated at 27 °C for 3–4 days and observed for bacterial growth. Colonies were counted, and the cfu g^{-1} calculated. Explants were sampled 12 and 16 weeks after transformation, and shoots were sampled at the 16 and 24 week stage.

Identification of LBA4404 using the polymerase chain reaction

Bacteria isolated from the plant tissue, with colony morphology similar to that of LBA4404, were confirmed as LBA4404 by using RAPDs. Isolation of chromosom DNA was carried out using the method of Armitage et al., (1988). Ten-ml cultures of *Agrobacterium* grown over a 48-h period were used to isolate 10–25 ng μl^{-1} of DNA. In order to determine that it would be possible to use RAPD's, ten random primers (1-GGTAGCACTC; 2-GGTCCTCAGG; 3-CAGTTCGAGG; 4-TACCGACACC; 5-TCGGAGTGGC; 6-ACTCAGGAGC; 7-CCACCGCCAC; 8-AGAGATGCCC; 9-CAGTTCTGGC; 10-CGTGCTAGCA; Pharmacia, Grosvenor Road, Herts, UK) were used to probe several isolates of *Agrobacterium* (See Figure 1 for strain type). Reactions were performed using 5 µl (25–50 ng) of DNA per sample with 5 µl of 10 × dNTP solution (2 mM), 5 µl of 10 × primer (0.2 mM), 5 µl of 10 × *taq* buffer, 0.10 (l of *taq* DNA polymerase (HT Biotechnology, Ditton Walk, Cambridge, UK) (5 U μl^{-1}), 29.9 µl of water and overlaid with mineral oil. Samples underwent 45 cycles of 92 °C, 1 min, of 40 °C, 3 mins and 72 °C, 1 min followed by one cycle of 72 °C for 5 mins. Samples were run on a 1.5% agarose gel at 110V for 4 hours followed by ethidium bromide staining for UV fluorescence.

Detection and confirmation of the presence of the binary vector

The presence or absence of the binary vector was detected by plating all *Agrobacterium* samples that had been re-isolated after 16 weeks from the plant tissue, onto LB containing 50 mg l^{-1} of kanamycin as well as LB with no antibiotics. Growth on LB but no growth on LB + Km (kanamycin) indicated that the binary vector had been lost. Growth on both plates suggested the presence of the binary vector. This was confirmed by using PCR with specific primers for NPTII (5'GCTATTCGGCTAT-

GACTG 3'and 3'GGGAGCGGCGATACCGTA 5'and GUS (5'GGTGGGAAAGCGCGTTACAAG 3'and 3'GTTTACGCGTTGCTTCCGCCA 5' (Pharmacia, Grosvenor Road, Herts, UK).

Plasmid DNA was isolated by using a plasmid kit (Qiagen, Max-Volmer-Stasse, Hilden, Germany). Double the recommended quantity of bacterial culture, suggested by the protocol, was necessary to yield 5 - 10 ng μl^{-1} of plasmid DNA. The reactions were carried out with 1 µl (5–10 ng) of DNA, 5 µl of 10 × dNTP solution (2 mM), 5 µl of 10 × 5'–3'primer, 5 µl of 10× 3'–5' primer, 5 µl of 10x *taq* buffer, 0.03 µl of *taq* polymerase (5U μl^{-1}), 28.97 µl of water and overlaid with mineral oil. Samples underwent 35 cycles of 92 °C, 1 min, of 62°C, 1.5 mins and 72 °C for 2 mins followed by a second stage of one cycle of 92 °C, 1 min, of 62 °C, 1.5 min and 72°C for 5 mins. Samples were stained as described as before.

Determination of the minimum bactericidal concentration (MBC)

The MBC for LBA4404 was determined prior to transformation by inoculating broths of LB and the respective tissue culture media (Table 1) with increasing concentrations of carbenicillin, ticaracillin and cefotaxime. Concentrations from 50 mg l^{-1} to 1000 mg l^{-1} were examined. The broths were then inoculated with 0.1 ml of a 24-h liquid culture of LBA4404 (3.7 × 10^6 cfu ml^{-1}). These were incubated overnight on a shaking waterbath at 27 °C and plated onto solid antibiotic-free LB. After 3–4 days further incubation, the plates were examined for growth. If the tubes were not turbid, and there was growth on the solid plates, then this implied that the antibiotics were bacteriostatic. If there was less growth than the control, then there was some bactericidal effect and the minimum concentration that yields no growth is the MBC value. Incorporation of antibiotic into the tissue culture medium, at a concentration of four times the MBC is recommended for elimination of bacteria in tissue culture (Falkiner pers, comm; Barrett and Cassells, 1994).

Results

Explant sampling for the presence of residual Agrobacterium

The results of sampling are given in Table 2. It can be seen that the percentage contamination remained high or increased between the 3 and 4-month stage on antibiotic medium. The colony forming units per gram varied greatly between samples, but values were often higher than the original inoculum, with residual bacteria as high as 10^9 cfu g^{-1}. Explants were no longer sampled after the 16-week stage because contamination became so high that *Solanum* explants senesced and died, and putative *Agrobacterium* began to exude from the tissues of *Brassica* and *Rubus*. This indicates that the antibiotics, at the concentrations shown, were not eliminating contamination.

Shoot sampling for the presence of residual Agrobacterium

As shoots were produced in *Brassica* explants within 6 weeks, it was decided to use this species to examine residual bacteria in shoots. The first 100 shoots produced were sampled, and 31% contained residual putative *Agrobacterium* at concentrations ranging from 5×10^1 up to 7.3×10^7 cfu g^{-1}. After a further 8 weeks (total of 24 weeks after transformation) on antibiotic medium, the shoots which had developed from the apical nodes were again sampled. No *Agrobacterium* was evident on the medium however, 24% gave positive results with a range of 6.2×10^1 to 8.8×10^8 cfu g^{-1}.

Preliminary Polymerase Chain Reaction results

DNA from LBA4404 and several other wild *Agrobacterium* species was probed with 10 random primers. Primer 10 gave no response, primers 1–4 and 9 gave only 1–2 bands, primers 5 and 6 gave ~ 3 bands and Primer 7 and 8 gave 4–5 bands. Figure 1 indicates the different banding patterns produced by LBA4404 and several of the wild isolates of *Agrobacterium* with primer 8. Five bands were produced by LBA4404, which occurred in all replicates (10 reactions) and had molecular weights between 2.35 and 0.4 kb. No other strain tested gave the same banding pattern therefore making it possible to distinguish LBA4404 using this method (Figure 1).

Identification of residual bacteria as LBA4404.

All colonies isolated were round, smooth, convex, cream-beige in colour, ~ 2 mm in diameter, and identical to those produced by the original stocks of LBA4404. Following DNA isolation, the putative *Agrobacterium* isolates from *Brassica*, *Rubus*, *Solanum* and LBA4404 (control) were probed with

Table 2. Percentage contamination of putative *Agrobacterium* (LBA4404) in explants (30–50 in each case) of *Brassica, Solanum* and *Rubus* after 12 and 16 weeks on antibiotic media. Selection was only applied in *Solanum*.

Explant	Antibiotic	Concentration mg l^{-1}	Contamination % ± SE	Number of weeks	Range cfu ml^{-1}
Brassica	Carbenicillin	500	46.0 ± 6	12	2.7×10^1 -46 × 10^6
			80.7 ± 15.5	16	4.8×10^4 - 1.3 × 10^9
Solanum	Cefotaxime	500	35.5 ± 13	12	6.1×10^2 - 1.9 × 10^9
	Kanamycin	250	35.0 ± 5	16	2.3×10^6 - 3.3 × 10^9
Rubus	Ticaracillin	125	97.5 ± 2.5	12	4.5×10^3 - 2.7 × 10^9
			90.4 ± 4.4	16	1.0×10^3 - 3.1 × 10^9

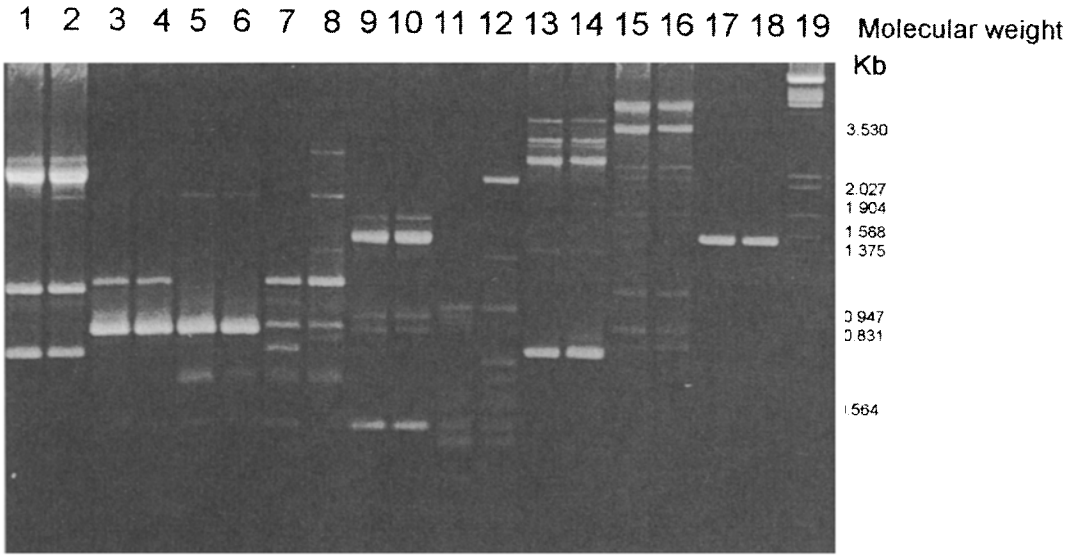

Figure 1. PCR of LBA4404 and wild strains of *Agrobacterium* using random primer 8. Lanes 1 and 2 indicate LBA4404; 3,4 - Ar2628; 5, 6 - Ar2629; 7, 8 - Ar9404; 9 to 18 - Uncharacterised strains of *Agrobacterium tumefaciens* isolated from various parts of the UK; 19, 20 - EcoR1/HindIII molecular weight marker.

primer 8. All patterns were the same as those for LBA4404 (Figure 2), indicating that the residual bacteria were LBA4404. The same result was obtained for the 16-week sampling in explants and the 24-week sampling in shoots.

Detection of the binary vector

The presence of the binary vector was detected in certain *Agrobacterium* anthers re-isolated from all three plant species (*Brassica Solanum* and *Rubus*), even when no selective pressure was applied. A large proportion (85% and 86%) of the contaminated explants of *Brassica* and *Rubus* contained *Agrobacterium* cells which still harboured the binary vector after 16 weeks in culture. All the contaminated explants of *Solanum* contained cells with the binary vector, as kanamycin is incorporated into the culture medium directly after transformation. The mean number of cfu g^{-1} of *Agrobacterium* isolated from these explants is given in Table 3. It can be seen that the mean number of cfu g^{-1} isolated on kanamycin medium is less than that on LB alone for *Brassica* and *Rubus*, indicating a loss of the binary vector, but is the same for *Solanum*, due to selective pressure.

A similar result was obtained for the shoots sampled at 16 weeks and 24 weeks. In this case however, only 59% (16 weeks) and 52% (24 weeks) of the contaminated tissue contained *Agrobacterium* cells which harboured the vector. The mean cfu g^{-1} is given in

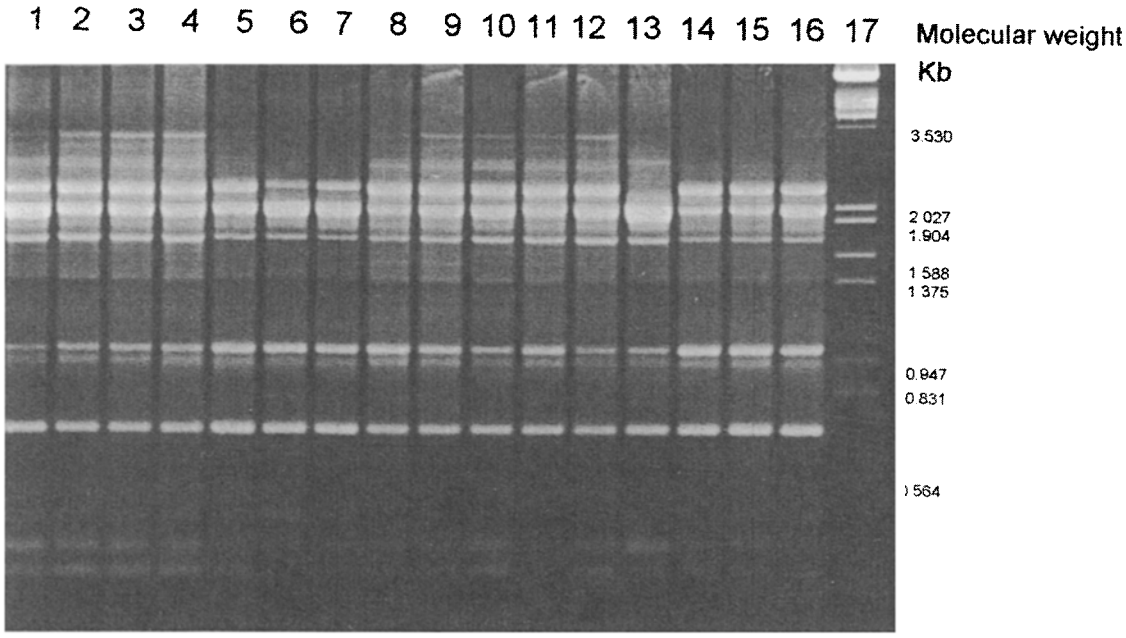

Figure 2. PCR of LBA4404 and 15 bacterial samples isolated from *Brassica*, 12 weeks after transformation using primer 8. Lane 1 indicates LBA4404.

Table 3. The mean number of cfu g^{-1} of *Agrobacterium* which grew on LB and LB + Km, isolated from explants (30–50 in each case) of *Brassica*, *Rubus*, and *Solanum*, and shoots of *Brassica*.

Time after transformation	Species	Mean no. of cfu g^{-1} LB	Mean no. of cfu g^{-1} LB + KM
Explants			
16 weeks	*Brassica*	$1.3 (\pm 0.6) \times 10^8$	$1.0 (\pm 0.8) \times 10^7$
16 weeks	*Rubus*	$1.7 (\pm 0.8) \times 10^8$	$2.4 (\pm 0.6) \times 10^7$
16 weeks	*Solanum*	$1.0 (\pm 0.3) \times 10^9$	$1.1 (\pm 0.3) \times 10^9$
Shoots			
16 weeks	*Brassica*	$1.6 (\pm 0.4) \times 10^8$	$9.0 (\pm 5.0) \times 10^6$
24 weeks	*Brassica*	$2.5 (\pm 0.6) \times 10^8$	$2.1 (\pm 0.8) \times 10^6$

Table 3. Again the lower number of cells on LB plus kanamycin is evident in comparison to that on LB with no antibiotics.

Confirmation of the presence of the binary vector

The growth of LBA4404 on kanamycin medium indicates the presence of the binary vector. This was confirmed using primers for the NPTII and GUS genes, the results of which are seen in Figure 3.

Minimum bactericidal concentration

After incubation in broths, none of the tubes were turbid. However, once inoculated onto LB plates, growth was evident from broths with 50–600 mg l^{-1} carbenicillin, 50–300 mg l^{-1} ticaracillin, and 50–100 mg l^{-1} cefotaxime. The growth was considerably less than that of the controls, thus indicating some bactericidal effect. The final MBC results are indicated in Table 4. It can be seen that the MBC's for both carbenicillin and

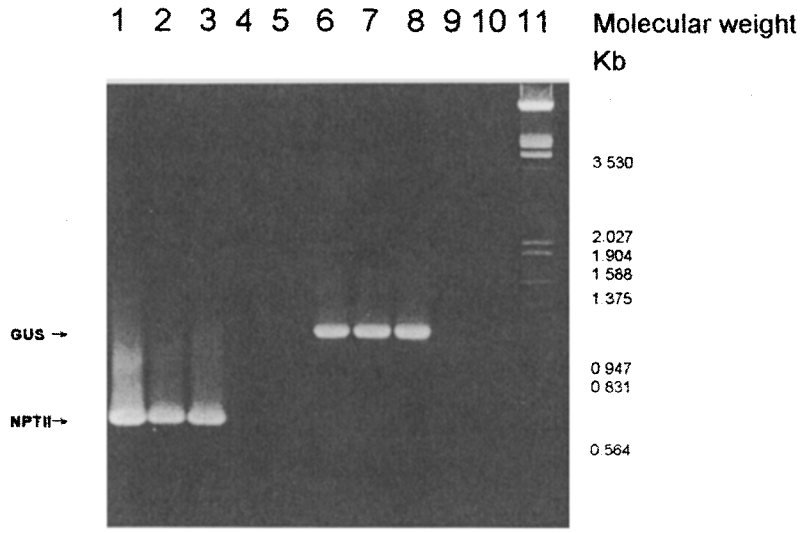

Figure 3. PCR of LBA4404, *Brassica*, *Rubus* and *Solanum* plasmid DNA with specific primers for NPTII and GUS.

Table 4. The minimum bactericidal concentration of cefotaxime, carbenicillin and ticaracillin to *Agrobacterium* (LBA4404) on LB and transformation medium.

Antibiotic	Medium	MBC mean mg l^{-1} ± SE
Carbenicillin	LB	733 ± 33.3
Carbenicillin	ADVB	600 ± 0.00
Ticaracillin	LB	400 ± 0.00
Ticaracillin	ADVRL	366 ± 33.3
Cefotaxime	LB	150 ± 0.00
Cefotaxime	ADVP	150 ± 0.00

LB - *Luria Bertani* broth (bacteriological medium)
ADVB - *Brassica* transformation medium
ADVRL - *Rubus* leaf disc transformation medium
ADVP - *Solanum* transformation medium

ticaracillin are actually higher than the concentrations used in the transformation systems, where 500 mg l^{-1} and 125 mg l^{-1} respectively are employed. The MBC for cefotaxime is 150 mg l^{-1}, which is lower than the concentration in the transformation system.

Discussion

Transformation of plants with *Agrobacterium* works on the basis that bacteria are eliminated with antibiotics during the regeneration stage. Transgenic plants to be released should not contain residual *Agrobacterium* as the possibility of gene escape, via the bacteria, exists. A second problem also arises. Molecular probes are often used in determining the presence of the 'integrated' T-DNA in the plant genome and the presence of residual *Agrobacterium* harbouring the binary vector may give false positives. The results of this study, using three model transformation systems, indicate the difficulty in eliminating *Agrobacterium* from cultures following transformation. The concentrations of antibiotics used in the case of carbenicillin and cefotaxime were the highest of values (500 mg l^{-1}) evident in the literature for transformation, yet they failed to eliminate contamination. In fact, the levels of *Agrobacterium*, in explants, increased and eventually became visible on the plates. Colony forming units of bacteria in shoots declined from 20 to 24 weeks, but still a considerable percentage (24%) of the shoot material was contaminated.

Examples only 250 mg l^{-1} of carbenicillin (da Camara Machado, 1992; Sarria et al., 1994) and 200–300 mg l^{-1} of cefotaxime (Maheswaran et al., 1992; Jacq et al., 1993; Pugliesi et al., 1993; De Bondt et al., 1994) are employed are commonly present in the literature. Studies carried out by Martin et al., (1990) and Valles and Lasa (1994) indicated that *Agrobacterium* was still visible on plates with these concentrations of antibiotics. This shows the inadequacy of some existing transformation systems.

The determination of the MBC's proved to be a good indicator as to one reason for *Agrobacterium* remaining present in the plant tissues, even after

6 months. The MBC's for both carbenicillin and ticaracillin are higher than the concentrations used in the transformation systems. It is evident from these results that levels of antibiotics higher than 500 mg l^{-1} would be necessary to eliminate *Agrobacterium*, but then the problem of phytotoxicity arises, therefore not enabling the use of higher concentrations. The antibiotics did prove to be bacteriostatic, therefore, containment of contamination in the explants or lower regions of shoot material may be possible. This would explain why *Agrobacterium* levels in the shoots were considerably less than those in the explant material. With frequent transferral of material onto fresh antibiotic medium, removing the shoots as they emerge and then by only using the apical nodes, up to 75 % appeared to be 'clean'. Although it is not possible to be completely sure that very low levels of contamination will be detected by any method of sampling, it does give a good indication.

The MBC for cefotaxime was lower than the concentration used for transformation. However contamination still remained. Previous studies (Barrett and Cassells, 1994) indicated that cefotaxime was not light sensitive and was stable under normal tissue culture growth room conditions for up to 22 days. Since transformed material was placed onto fresh medium every 10 days, stability of the antibiotic was not a problem. It has been recommended (Falkiner, pers. comm.; Barrett and Cassells, 1994) that at least 4 times the MBC of the antibiotic is incorporated into the tissue culture medium for successful bacterial elimination. This value would be higher than the cefotaxime concentration used in the system, and could therefore explain the failure to eliminate *Agrobacterium*. The possible use of combinations of antibiotics may prove to be the better alternative (Horsch and King, 1983; Leifert et al., 1991).

A more important problem which has arisen as a result of these experiments is the retention of the binary vector after 6 months without any selection pressure. Over half of the contaminated shoot material harboured *Agrobacterium* cells containing the binary vector, with, on average, 2.1×10^6 to 1.1×10^9 cfu g^{-1} of tissue. Here is where the possibility of gene escape arises. The presence of the disarmed *Agrobacterium* in the tissue would not be a problem if the binary vector had been 'lost', but now its survival and spread are real possibilities. As far back as 1977, Kerr et al., and Genetello et al., showed that the Ti-plasmid could be easily conjugated to other *Agrobacterium* strain. Mogilner et al., (1993) reported that a disarmed *Agrobacterium* containing a Ti-plasmid with a dimeric cDNA of *Citrus exocortis* viroid (CEVd) survived and retained the plasmid after three months in agro-infected plants. Maintenance of the binary vector long enough for the disarmed strain to come into contact with a wild strain of *Agrobacterium* could conceivably lead to gene escape to the wild strain. The only method of guaranteeing this does not occur is to ensure that no *Agrobacterium* is present in transgenic plants prior to their release. A second problem arising is one of false positives. The use of molecular methods to indicate transformation by using primers for the NPTII and GUS genes (Millam, 1989; Hamill et al., 1990) relies on the absence of residual *Agrobacterium* to give an accurate result. Molecular analysis of plants contaminated with *Agrobacterium* harbouring the binary vector may indicate that the plants have been transformed, while in actual fact the positive result is due to the presence of the vector and not the integrated T-DNA. Hamill et al. (1990) suggested the use of a primer to amplify an area on the Ti-plasmid outside the T-DNA in order to indicate the presence of contaminating *Agrobacterium*.

The fact that bacteria remain latent during tissue culture (Cassells, 1991; Leifert et al., 1991; Bastaiens et al., 1983) stresses the need for sampling the tissue and not just examining at a visible level. Latency may occur as a result of low bacterial numbers or due to unfavourable environmental conditions (Hayward, 1974). Many of the organisms inhabiting plant tissues are often inhibited by the components of the culture medium, such as high salt or sucrose concentration and pH (Cassells et al., 1988). This results in the multiplication of the bacteria with the plant tissue without any evidence on the growth medium. This occurred in the shoot samples of *Brassica* in which no *Agrobacterium* was evident but up to 24% of the material was still contaminated. Sampling the material for bacteria, in this case *Agrobacterium*, will, in most cases, indicate contaminated material which then may be removed and thus prevent the possibility of gene escape occurring in this manner.

Conclusions

The results of all these experiments indicate the need for risk assessment studies. The problem of gene escape may arise as a result of transformation, through carryover of residual *Agrobacterium* within the plant tissue. However, the procedure of sampling during

the regeneration process and removing any contaminated material at an early stage reduces drastically the possibility of gene escape occurring from the disarmed strain of *Agrobacterium* used for transformation. It also eliminates the possibility of giving 'false transgenics' using molecular techniques to indicate the presence of marker genes such as NPTII and GUS genes.

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References

Armitage P, Walden R & Draper J (1988) Vectors for transformation of plant cells using *Agrobacterium*. In: Draper J, Scott R, Armitage P & Walden R (eds) Plant Genetic Transformation and Gene Expression: A Laboratory Manual (pp 58–60). Blackwell Scientific Pubs., Oxford

Barrett C & Cassells AC (1994) An evaluation of antibiotics for the elimination of *Xanthomonas campestris* pv. *pelargonii* (Brown) from *Pelargonium* × *domesticum* cv. Grand Slam explants *in vivo*. Plant Cell Tiss. Org. Cult. 36: 169–175

Barghchi M, Turgut K, Scott R & Draper J (1994) High frequency transformation from cultured cotyledons of *Arabadopsis thaliana* ecotypes "C24" and "Landsberg *erecta*". Plant Growth Regulations 14: 61–67

Bastaeiens L, Maene L, Harbaori Y, Van Sumere C, Van de Castelle KL & Debergh PC(1983) The influence of antibacterial products on plant tissue cultures. Med. Fac. Landbouwv. Rijsuniv. Gent. 48: 13–24

Bertram J, Stratz M & Durre P(1991) Natural transfer of conjugative transposon Tn916 between gram - positive and gram - negative bacteria. J. Bacteriol. 173(2): 443–448

da Camara Machado ML, da Camara Machado A, Hanzer V, Weiss H, Regner F, Steinkellner H, Mattanovich D, Plail R, Knapp E, Kalthoff B & Katinger H (1992) Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of Plum Pox Virus. Plant Cell Rep. 11: 25–29

Cassells AC, Harney MA, Carney BF, Mc Carthy E & Mc Hugh A (1988) Problems posed by cultivable bacteria and the endophytes in the establishment of axenic cultures of *Pelargonium* × *domesticum*: The use of *Xanthomonas pelargonii* specific ELISA, DNA probes and culture indexing in the screening of antibiotic treated and untreated donor plants. Acta Hort. 225: 153–162

Cassells AC (1991) Problems in tissue culture: culture contamination. In: Debergh PC & Zimmerman RH (eds) Micropropagation (pp 31–44). Kluwer Acad. Pubs, Netherlands

Confalonieri M, Balestrazzi A & Bisoffi S (1994) Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*. Plant Cell Rep. 13: 256–261

Dale PJ, Irwin JA & Scheffler JA (1993) Review: The experimental and commercial release of transgenic crop plants. Plant Breeding 111: 1–22

De Bondt A, Eggermont K, Druart P, De Vil M, Goderis I, Vanderleyden J & Broekaert W (1994) *Agrobacterium*-mediated transformation of apple (*Malus* × *domestica* Borkh.): An assessment of factors affecting gene transfer efficiency during early transformation steps. Plant Cell Rep. 13: 587–593

Draper J, Scott R & Hamil J(1988) Transformation of dicotyledonous plant cells using the Ti plasmid of *Agrobacterium tumefaciens* and the Ri plasmid of *A. rhizogenes*. In: Draper J, Scott R, Armitage P and Walden R (eds) Plant Genetic Transformation and Gene Expression: A Laboratory Manual (pp71–132). Blackwell Scientific Pubs, Oxford

Eapen S & George L (1994) *Agrobacterium tumefaciens* mediated gene transfer in peanut (*Arachis hypogaea* L.). Plant Cell Rep. 13: 582–586

Genetello C, Van Larebeke N, Holsters M, De Picker A, Van Montagu M & Schell J (1977) Ti plasmids of *Agrobacterium* as conjugative plasmids. Nature 260: 561–562

Gonsalves C, Xue B, Yepes M, Fuchs M, Ling K, Namba S, Chee P, Slightim JL & Gonsalves D (1994) Transferring cucumber mozaic virus-white leaf strain coat protein gene into *Cucumis melo* L. and evaluating transgenic plants for protection against infections. J. Am. Soc. Hort. Sci. 119(2): 345–355

Graham J & Mc Nicol R (1990) Plantlet regeneration and genetic transformation in soft fruit species. Acta Hort. 280: 517–522

Grierson D & Covey S (1984) Genetic colonisation of plants by *Agrobacterium*. In: Plant Molecular Biology (pp 112–125). Blackie & Son Pubs., Glasgow

Hamill J, Rounsley S, Spencer A, Todd G & Rhodes M (1990) The use of the polymerase chain reaction to detect specific sequences in transformed plant tissues. In: Nijkamp H J S, van der Plas L H W & van Aartijk (eds) Progress in Plant Cellular and Molecular Biology (pp 183–188). Kluwer Acadmic, Dordrecht.

Hassan M, Swartz H, Inamine G & Mullineaux P (1993) *Agrobacterium tumefaciens* - mediated transformation of several *Rubus* genotypes and recovery of transformed plants. Plant Cell Tiss. Org. Cult. 33: 9–17

Hayward AC (1974) Latent infections by bacteria. Ann. Rev. Phytopath. 12: 87–97

Heinemann J & Sprague G (1989) Bacterial conjugative plasmids mobilise DNA transfer between bacteria and yeast. Nature 340: 205–209

Higgins E, Hulme J & Shields R (1992) Early events in transformation of potato by *Agrobacterium tumefaciens*. Plant Science 82: 109–118

van der Hoeven C, Dietz A & Landsmann J (1994) Variability of organ-specific gene expression in transgenic tobacco plants. Trans. Res. 3: 159–165

Horsch R & King K (1983) A covert contaminant of cultured plant cells: elimination of a *Hyphomicrobium* sp. from culture of *Datura innoxia* (Mill.). Pl. Cell Tiss. Org. Cult. 2: 21–28

Howe GT, Goldfarb B & Strauss SH (1994) *Agrobacterium* - mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants. Plant Cell Tiss. Org. Cult. 36: 59–71

Hulme J, Higgins E & Shields R (1992) An efficient genotype - independent method for regeneration of potato plants from leaf tissue. Plant Cell Tiss. Org. Cult. 3: 161–167

Jacq B, Lesobre O, Sangwan S & Sangwan - Noreel S (1993) Factors influencing T - DNA transfer in *Agrobacterium* - mediated transformation of sugarbeet. Plant Cell Rep. 12: 621–624

Jefferson R A (1987a) GUS gene fusion system user's manual. Plant Breeding Institute, Cambridge version 1.21

Jefferson R A (1987b) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5(4): 387-405

Jones JB & Raju BC (1988) Systemic movement of *Agrobacterium* tumefaciens in symptomless stem tissue of *Chrysanthemum morifolium*. Plant Disease 72: 51–54

Kerr A, Manigault P & Tempe J (1977) Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium*. Nature 265: 560–561

Leifert C, Camotta H, Wright S, Waites B, Cheyene V & Waites WM (1991) Elimination of *Lactobacillus plantarum*, *Corynebacterium* spp., *Staphlococcus saprophyticus* and *Pseudomonas paucimobilis* from micropropagated *Hemercallis*, *Choisya* and *Delphinium* cultures using antibiotics. J. App. Bact. 71: 307–330

Lilley A, Fry J, Day J & Bailey M (1994) *In situ* transfer of an exogenously isolated plasmid between *Pseudomonas* spp. in sugarbeet rhizosphere. Microbiology140: 27–33

Lorenz M & Wackernagel W (1991) High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorus source. Applied and Environmental Microbiology 57(4:): 1246–1251

Maheswaran G, Welander M, Hutchinson JF, Graham MW & Richards D (1992) Transformation of apple rootstock M26 with *Agrobacterium tumefaciens*. J. Plant Physiol. 139: 560–568

Martin GC, Miller AN, Castle LA, Morris JW & Dandekar AM (1990) Feasibility studies using the β-glucuronidase as a gene fusion marker in apple, peach and radish. J. Am. Soc. Hort. Sci. 115(4): 686–691

Millam S (1989) *Agrobacterium* - mediated transformation systems of *Brassica* species. Aspects of Appl. Biol. 23: 23–30

Mlynarova L, Bauer M, Nap J & Pretova A (1994) High efficiency *Agrobacterium* - mediated gene transfer to flax. Plant Cell Rep. 13: 282–285

Mogilner N, Zutra D, Gafny R & Bar - Joseph M (1993) The persistence of engineered *Agrobacterium tumefaciens* in agroinfected plants. Molecular Plant - Microbe Interactions 6(50): 673–675

Moloney M, Walker J & Sharma K (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep. 8: 238–242

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco. Physiol. Plantarum 15: 47–497

Pugliesi C, Biasini MG, Fambrini M & Baroncelli S (1993) Genetic transformation by *Agrobacterium* in the interspecific hybrid *Helianthus annuus* × *Helianthus tuberosus*. Plant Sci. 93: 105–115

Radke S, Andrews B, Moloney M, Crouch M, Kridl J & Knauf V (1988) Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. Theor. Appl. Gen. 75: 685–694

Sarria R, Calderon A, Thro AM, Torres E, Mayer J & Roca WM (1994) *Agrobacterium*-mediated transformation of *Stylosanthes guianensis* and production of transgenic plants. Plant Sci. 96: 119–127

Schafer A, Kalinowski J, Simon R & Seep - Feldhaus A (1990) High - frequency conjugal plasmid transfer from gram - negative *Escherichia coli* to various gram - positive Coryneform - bacteria. J. Bacteriol. 172(3): 1663–1666

Tarbah FA & Goodman RN (1987) Systemic spread of *Agrobacterium tumefaciens* Biovar 3 in the vascular system of grapes. Phytopath. 77: 915–920

Valles MP & Lasa JM (1994) *Agrobacterium*-mediated transformation of commercial melon (*Cucumis melo* L., cv. Amarillo Oro). Plant Cell Rep. 13: 145–148

Wenzler H, Mignery G, May G & Park W (1989) A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. Plant Sci. 63: 79–85