Transfer of the *Agrobacterium tumefaciens* TI Plasmid to Avirulent Agrobacteria and to Rhizobium *ex planta*

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SUMMARY

A mutant of *A. tumefaciens* strain B6S3, carrying the R factor RP4, was able to transfer its TI plasmid to various avirulent Agrobacterium strains and to a strain of Rhizobium. Strains carrying the TI(B6S3) plasmid were selected by their ability to utilize octopine. The isolates were able to induce tumours and exclude phage API. The tumours induced on *Kalanchoë daigremontiana* were rough and contained octopine. It was concluded that octopine utilization, phage exclusion, induction of rough tumours and synthesis of octopine in the tumours are determined by the TI(B6S3) plasmid. Tumorigenicity was determined by this plasmid both in Agrobacteria and in Rhizobium, suggesting that all the genes necessary for tumour induction are plasmid-borne.

INTRODUCTION

The plant tumour, crown gall, can be induced on many dicotyledonous plants by inoculation of a wound site with *Agrobacterium tumefaciens* (Smith & Townsend) Conn. The mechanism whereby the bacterium transforms normal cells into tumour cells is unknown (Drlica & Kado, 1975; Lippincott & Lippincott, 1975; Schilperoort & Bomhoff, 1975). It seems likely that genes located on a large bacterial plasmid (TI) are involved in the tumorigenicity since all virulent Agrobacterium strains contain the plasmid, while almost all avirulent strains lack it (Zaenen *et al.*, 1974). Virulent strains become avirulent upon 'curing' of the plasmid (Van Larebeke *et al.*, 1974), while avirulent strains become virulent when they receive it from a virulent donor strain (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975).

Transfer of the TI plasmid has previously been reported only in mixed infections on the plant. In this paper we describe the *ex planta* transfer of it from a virulent, octopine-degrading Agrobacterium strain, harbouring the R factor RP4 (Datta *et al.*, 1971; Datta & Hedges, 1972), to several avirulent recipient strains of Agrobacterium and to Rhizobium. The localization of some markers previously described as plasmid-borne, e.g. virulence, ability to utilize octopine or nopaline (Schilperoort *et al.*, 1975; Watson *et al.*, 1975; Bomhoff *et al.*, 1976), sensitivity to agrocin Kerr84 (Engler *et al.*, 1975) and exclusion of phage API (Van Larebeke *et al.*, 1975; Schell, 1975) is discussed.

METHODS

Bacterial strains. Wild-type strains of *Agrobacterium tumefaciens* were: B6; B6S3; S1005; TR-1; TR-6; C58; Ach-5; Ach-5-C3; 0362; 3/1; Kerr14 and T37 obtained from Dr J. Schell (Gent, Belgium); Kerr84 from Dr A. Kerr (Glen Osmond, Australia); E III 9.6.1 from Laboratory of Microbiology, T.H.Delft, The Netherlands; ID135 AO from Dr C. I. Kado

Table 1. *Strains of A. tumefaciens isolated in our laboratory*

LBA strain*	Origin	Markers	Known plasmids carried
33	B6S3 × LBE1316		RP4; TI(B6S3)
52	B6S3 (mutagenized with NTG)	Met⁻Ile⁻	TI(B6S3)
57	LBA52 × LBA33	Met⁻Ile⁻	RP4; TI(B6S3)
202	C58 (heat treated at 37 °C)		None
270†	C58 (spontaneous mutant for utilization of octopine)	Utilization of nopaline and octopine	TI(C58)
601†	LBA202 × LBA57		RP4; TI(B6S3)

* LBA, Lugdunum Batavorum Agrobacterium.
† Isolation and characterization described in this paper.

(Davis, U.S.A.); and NCPPB396 from the National Collection of Plant Pathogenic Bacteria (Harpenden, Hertfordshire). Mutants of *A. tumefaciens* isolated in our laboratory are listed in Table 1. *Rhizobium trifolii* strain 5 was obtained from the Rothamsted Collection of Rhizobium (Harpenden). *Escherichia coli* K12 *pro met* (λ) (RP4) (LBE1316 in our collection) was received as strain J53(RP4) from Dr M. van Montagu (Gent, Belgium). All incubations were carried out at 29 °C.

Media. The rich medium (NB) contained (g l⁻¹): Difco nutrient broth, 8·0; Difco yeast extract, 5·0; NaCl, 5·0. Medium LC, used in phage typing and tests of sensitivity to agrocin S1005, contained (g l⁻¹): Difco tryptone, 10·0; Difco yeast extract, 5·0; NaCl, 8·0. The minimal medium (SM) was that of Klapwijk *et al.* (1975); it was also used in tests of sensitivity to agrocin Kerr84 and agrocin NCPPB396. When SM was used for the selection of octopine-utilizing strains, ammonium sulphate was omitted (SM-N) and octopine was added to a final concentration of 100 mg l⁻¹. Rhizobia were grown on YMB as a rich medium that contained (g l⁻¹): mannitol, 10·0; Difco yeast extract, 0·4; NaCl, 0·1; MgSO₄.7H₂O, 0·2; K₂HPO₄, 0·5; pH 6·8. The minimal medium for Rhizobium (RMM) contained (g l⁻¹): mannitol or glucose, 10·0; KNO₃, 0·6; CaCl₂.2H₂O, 0·1; MgSO₄.7H₂O, 0·25; KH₂PO₄, 1·0; K₂HPO₄, 1·0; FeCl₃.6H₂O, 0·01; and (μg l⁻¹): MnSO₄.H₂O, 20; ZnSO₄.7H₂O, 20; CuSO₄.5H₂O, 20; H₃BO₃, 20; CoCl₂, 2; Na₂MoO₄, 20; biotin, 10; calcium pantothenate, 10; thiamin, 10. When required, media were solidified with 1·8 % (w/v) Difco Bacto-agar, or, when testing for utilization of octopine, with 2·6% (w/v) Difco Special Agar Noble.

Mutagenesis and mutant isolation. N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis was as described by Klapwijk *et al.* (1975). Mutants were isolated after replica plating.

Plasmid transfer. Exponential-phase cultures of LBA57 and LBA202 in NB or SM, each containing 10⁹ bacteria ml⁻¹, were mixed and 0·1 ml was plated on SM plus carbenicillin (100 μg ml⁻¹), SM-N plus octopine or SM-N plus octopine plus carbenicillin (100 μg ml⁻¹). After 2 days a layer of confluent growth had formed on the plates; from these a loopful of culture was inoculated into SM-N plus octopine. After these liquid cultures had reached the stationary phase, bacteria were plated, purified three times by single colony isolation and then characterized.

Phage sensitivity. Phage immunity patterns were determined by spotting small drops of phage lysates on double agar layers, consisting of bottom layers of 20 ml 1·8 % agar and top layers of 4 ml 0·6 % soft-agar, containing exponential-phase cultures of the bacteria to be tested. Exclusion of phage AP1 was determined as described by Klapwijk *et al.* (1976). U5, a phage with a broad host-range specific for bacteria harbouring a plasmid of incompatibility class P, was obtained from Dr J. Schell (Gent, Belgium), as were phages S1, S2, S3, S5, S6 and PS8vir.

Agrocin sensitivity. Sensitivity to the agrocins produced by Agrobacterium strains SI005, TR-I, TR-6, Kerr84 and NCPPB396 was determined as described by Engler *et al.* (1975). For the determination of the sensitivity to agrocins of SI005, TR-I and TR-6 LC medium was used, while for that to the other agrocins SM medium was used.

Utilization of octopine and nopaline. This was determined by growing the bacteria in SM medium containing octopine or nopaline at a final concentration of 100 mg l^{-1}. After 24 h incubation a small drop of the culture was spotted on to Whatman 3MM paper and stained for guanidines with the phenanthrenequinone reagent (Yamada & Itano, 1966).

Determination of octopine uptake was as described by Klapwijk *et al.* (1976).

Assay for virulence. Tumours were induced on stems of *Kalanchoë daigremontiana* (Hamet & Perrier), and *Nicotiana tabacum* var. White Burley as described previously (Schilperoort, 1969; Bomhoff *et al.*, 1976). Tumours were induced on stems of *Helianthus annuus* var. giganteus by puncturing the stems with a sterile wooden pricker, the top of which had previously been dipped into a colony of the bacterium to be tested.

Detection of guanidines in tumours was as described by Schilperoort & Bomhoff (1975).

Test of nodulation and nitrogen fixation. Enclosed cultures on seedling agar were carried out according to Vincent (1970); 12 replicates on both *Trifolium pratense* cv.5123 and *Trifolium parviflorum* (kindly provided by Dr P. S. Nutman, Rothamsted, Harpenden) were examined for each test. Nodulation was scored after 4 weeks. Acetylene reduction by intact plants was measured in air containing 10 % acetylene (Planqué & Van Brussel, 1976); gas chromatography was carried out according to Citernesi *et al.*, (1976).

Plasmid isolation. Plasmids were detected on alkaline sucrose gradients, using alkaline lysates of Agrobacteria (Ledeboer *et al.*, 1976) or neutral lysates of Rhizobia (Nuti, unpublished results) labelled with [³H]thymidine. The plasmid nature of the peaks was always confirmed by renaturation kinetics (Ledeboer *et al.*, 1976).

BL-test for the production of 3-ketoglycosides was as described by Bernaerts & De Ley (1963).

Chemicals. NTG was obtained from Pfaltz & Bauer, New York, U.S.A.; carbenicillin from Beecham, Heppignies, Belgium; D(+)-octopine from Sigma. Nopaline was extracted from plant tumours (Bomhoff *et al.*, 1976). All other chemicals were of standard analytical grade.

RESULTS

Plasmid transfer

Overnight cultures of LBA52, LBA57 and LBA202 grown in NB were diluted 1:20 in fresh medium and grown for 3 to 4 h; they were then plated separately or mixed on the selective media, SM-N plus octopine, SM plus carbenicillin and SM-N plus octopine plus carbenicillin, and scored after 2 days incubation (Table 2). Only when both LBA57 and LBA202 were plated on the selective media was a thick layer of confluent growth observed. Plating of LBA202 in the presence or absence of LBA52 on SM-N plus octopine resulted in light growth, probably because the medium provided enough nitrogen to sustain some 'background' growth. From the above plates a loopful of culture was inoculated into SM-N plus octopine. Only when inocula were taken from plates on which both LBA57 and LBA202 were plated was growth observed in the liquid culture. Bacteria were isolated from stationary-phase cultures, purified three times by single colony isolation and then characterized.

Table 2. *Growth characteristics on selective media*

	SM + carbenicillin	SM-N + octopine	SM-N + octopine + carbenicillin
LBA52	−	−	−
LBA57	−	−	−
LBA202	−	±	−
LBA52 + LBA202	−	±	−
LBA57 + LBA202	+	+	+

−, No growth; +, good growth; ±, light growth.

Properties of the exconjugants

Since all the exconjugants (75 from 15 independent experiments) had the same properties, those of only one (LBA601) are recorded (Table 3). The phage and agrocin immunity pattern of LBA601 is identical to that of LBA202. Unlike LBA202, LBA601 is virulent, excludes phage AP1 and metabolizes octopine. Moreover it has all known RP4 markers i.e. resistance to carbenicillin, kanamycin and tetracycline and sensitivity to the P-plasmid-specific phage U5. Alkaline sucrose gradients of a lysate of LBA601 revealed that it contains RP4 and a plasmid that is as large as the TI plasmid. Renaturation experiments showed that the large plasmid was identical to the TI(B6S3) plasmid. (The wild-type strain harbouring the plasmid is indicated in parentheses.) The restriction pattern of the plasmid was similar to that of the TI(B6S3) plasmid, but different from the TI(C58) plasmid (J. Schell, personal communication). This showed that we had transferred the TI(B6S3) plasmid *ex planta* from LBA57 to LBA202 and that genes involved in the breakdown of octopine and in exclusion of phage AP1 are located on it. LBA601, like LBA57, induced tumours on *Kalanchoë daigremontiana* that contain octopine and have a rough surface with roots all over them, while C58, the parent strain of LBA202, induces tumours which contain nopaline and have a smooth surface with roots only at the bottom of the tumour (Fig. 1). Apparently the plasmid determines the type of tumour that will develop irrespective of the chromosomal background.

Transfer of the TI plasmid to other recipients and properties of the exconjugants

The TI(B6S3) plasmid was transferred by the procedure described above to Ach-5-C3, a derivative of Ach-5 cured from its TI plasmid by growth at 37 °C. This host also acquired the abilities to induce rough tumours containing octopine on *Kalanchoë daigremontiana* and to break down octopine. We were unable to transfer the plasmid to *A. tumefaciens* strains S1005, TR-1 and TR-6, probably because these strains produce an agrocin to which the donor is sensitive.

The TI(B6S3) plasmid was transferred from LBA57 to *Rhizobium trifolii* strain 5. Rhizobium, like all Agrobacterium recipients, gained the ability to induce tumours and to metabolize octopine (Table 4). The exconjugant (LPR501) was shown to be a *R. trifolii* strain by its inability to grow on NB and LC, its negative response in the BL-test, and its ability to form nitrogen-fixing nodules on *Trifolium pratense*. Unexpectedly, LPR501 was not able to nodulate *T. parviflorum*. Alkaline sucrose gradients revealed that *R. trifolii* strain 5 contains a plasmid of its own which is considerably larger than the TI(B6S3) plasmid (Nuti, unpublished results). The exconjugant, however, only carries a plasmid of the size of the TI(B6S3) plasmid. Thus, either *R. trifolii* had lost its own large plasmid after introduction of the TI(B6S3) plasmid, or a recombinant plasmid was formed.

Fig. 1. Virulence tests on *Kalanchoë daigremontiana* with (left) B6S3, (centre) LBA601 and (right) C58. Note the tumour morphology: rough tumours with roots all over them for B6S3 and LBA601, smooth tumours with root formation only at the bottom of the tumour for C58.

Table 3. *Properties of* LBA601 *compared with donor* (LBA57) *and recipient* (LBA202) *strain and some reference strains*

	Strain					
	B6S3	LBA57	LBA202	LBA601	C58	LBA270
Growth on SM	+	−	+	+	+	+
Growth on SM-N + octopine	+	−	−	+	−	+
Utilization of octopine	+	+	−	+	−	+
Utilization of nopaline	−	−	−	−	+	+
Sensitivity to:						
Phage S1	−	−	−	−	−	−
Phage S2	−	−	+	+	+	+
Phage S3	+	+	−	−	−	−
Phage S5	−	−	+	+	+	+
Phage S6	−	−	+	+	+	+
Phage PS8vir	+	+	−	−	−	−
Agrocin s1005	+	+	−	−	−	−
Agrocin NCPPB396	−	−	−	−	+	+
Agrocin Kerr84	−	−	−	−	+	+
Virulence	+	+	−	+	+	+
Guanidines in tumour	octopine	octopine		octopine	nopaline	nopaline
Tumour morphology	rough	rough		rough	smooth	smooth
Sensitivity to phage AP1	+	+	+	+	+	+
Exclusion of phage AP1	+	+	−	+	+	+
Presence of a large plasmid	+	+	−	+	+	+
Resistance to:						
Carbenicillin (100 µg ml^{-1})	−	+	−	+	−	−
Kanamycin (50 µg ml^{-1})	−	+	−	+	−	−
Tetracycline (2 µg ml^{-1})	−	+	−	+	−	−
Sensitivity to phage U5	−	+	−	+	−	−

Table 4. *Properties of the exconjugant R. trifolii* LPR501 *compared with the donor A. tumefaciens* LBA57 *and recipient R. trifolii* 5

	A. tumefaciens LBA57	R. trifolii 5	R. trifolii LPR501
Growth on NB	+	−	−
Growth on LC	+	−	−
BL-test	+	−	−
Utilization of octopine	+	−	+
Sensitivity to:			
Phage S3	+	−	−
Phage PS8vir	+	−	−
Agrocin S1005	+	−	−
.Agrocin NCPPB396	−	−	−
Virulence*	+	−	+
Guanidines in tumour	octopine		octopine
Tumour morphology	rough		rough
Infectivity on T. pratense	−	+	+ †
Effective symbiosis with T. pratense		+	+
Infectivity on T. parviflorum	−	+	−
Effective symbiosis with T. parviflorum		−	
Presence of a large plasmid	+	+	+
Estimated mol. wt of plasmid (Mdalton)	120	250–350	120

    * Tested on *Helianthus annuus, Nicotiana tabacum* and *Kalanchoë daigremontiana.*
    † No octopine detected in nodules.

Virulent strains as recipients in transfer-experiments

When avirulent, plasmid-less strains of *A. tumefaciens* like LBA202 and S1005 were plated alone on SM-N plus octopine light growth was observed, but when virulent, nopaline-degrading strains (C58, E III 9.6.1, 0362, 3/1, Kerr14, T37 and ID135 AO) were plated on the same medium a number of colonies always appeared in a background of light growth. The bacteria in these colonies were mutants able to break down nopaline as well as octopine. In these mutants the permease for the uptake of octopine and nopaline was constitutive, whereas it had to be induced by nopaline in the wild-type strains (Klapwijk, unpublished results).

When virulent, nopaline-degrading strains were used as recipients for the TI(B6S3) plasmid, strains were found that were able to metabolize both octopine and nopaline. Unlike avirulent recipients which received the TI(B6S3) plasmid, these strains induced smooth, nopaline-containing tumours on *Kalanchoë daigremontiana.* Thus, the isolates were probably not exconjugants, but spontaneous mutants of the type described above.

DISCUSSION

Transfer of the TI(B6S3) plasmid is observed only if relatively large numbers of parental bacteria are brought together on a solid medium and exconjugants can only be isolated from the layer of confluent growth. Thus the frequency of transfer cannot be determined quantitatively. In several experiments, confluent growth was less heavy and 10 to 20 clearly distinguishable recombinant colonies were observed. As 10^8 parental bacteria were added per plate the frequency of transfer is about 10^{-7}, or less if the parental bacteria need to multiply before transfer is possible.

The role of the plasmid RP4 in the *ex planta* transfer of the TI plasmid is uncertain. Our results suggest that RP4 is involved in the mobilization of the plasmid. All the strains which had received the TI plasmid, also received RP4. Also, transfer of the TI plasmid from strain

LBA52, which is isogenic with LBA57 except for its lack of RP4, was not observed. Transfer of the TI plasmid of some nopaline-utilizing strains has been reported to occur in the absence of RP4 in a mixed infection on the plant (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975). The TI plasmids may have fertility functions which are only expressed in the presence of some plant components.

Our results clearly demonstrate that genes involved in the metabolism of octopine are located on the TI(B6S3) plasmid. Klapwijk *et al.* (1976) suggested that at least three genes are involved in the metabolism of octopine: a regulator gene (*uadR*), a permease gene (*uadB*) and an oxidase gene (*uadA*). Permease-tests with LBA601 revealed that at least the regulator gene and the permease gene are located on the TI(B6S3) plasmid (Klapwijk, unpublished results). Since Rhizobium could utilize octopine after introduction of the TI(B6S3) plasmid, we conclude that the oxidase gene is of plasmid origin, unless such a gene was present in Rhizobium. Our results show that genes which determine the morphology of the tumour and the synthesis of octopine or nopaline in the tumour are also located on the TI plasmid.

Strain C58 excludes phage AP1, while the cured derivative, LBA202, does not (Van Larebeke *et al.*, 1975; Schell, 1975). We have shown that LBA601 also excludes phage AP1, therefore a locus responsible for the exclusion of phage AP1 is present both on the TI(B6S3) plasmid and the TI(C58) plasmid. A locus for sensitivity to agrocin Kerr84 is located on the TI(C58) plasmid (Engler *et al.*, 1975), but the locus for sensitivity to agrocin S1005 seems to be chromosomal, since LBA601 is insensitive to the agrocin, whereas the donor strain LBA57 is sensitive. While C58 can be cured of its plasmid by growth at 37 °C, LBA601 does not lose its plasmid by growth at the same temperature. Thus, as was stated by Watson *et al.* (1975), a locus on the TI(C58) plasmid is probably responsible for curing by growth at 37 °C.

Rhizobium trifolii strain 5, like Agrobacterium recipients, acquired the ability to break down octopine and to induce tumours on introduction of the TI(B6S3) plasmid. Although the reason for loss of infectivity on *T. parviflorum* remains obscure, the exconjugant had not lost the ability to form effective nodules on *T. pratense*. The observation that Rhizobium became tumorigenic on the introduction of the TI plasmid suggests that the plasmid DNA of strain B6S3 itself or some of its gene products are sufficient for the induction of tumours.

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