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Agrobacterium tumefaciens Mutants Affected in Crown Gall Tumorigenesis and Octopine Catabolism

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Mutants of *Agrobacterium tumefaciens* which affect virulence or the ability to catabolize octopine were isolated after Tn*5*-induced mutagenesis. Of 8,900 colonies tested, 7 mutants with Tn*5* insertions in a specific region of the Ti plasmid unable to catabolize octopine were isolated. Thirty-seven mutants affected in tumorigenesis resulted from insertions in the Ti plasmid and the *Agrobacterium* chromosome. Of these mutations, 12 were chromosomal and 25 mapped on the plasmid. Twenty-three mapped within a 20-megadalton region, which is distinct from the Ti plasmid sequences found stably integrated into the plant cell genome (T-deoxyribonucleic acid). Included in these were mutants that were either avirulent or produced tumors with unusual morphologies. Three mutants contained insertions in the T-deoxyribonucleic acid. These three mutants incited tumors which synthesized octopine but had an altered morphology due to either extensive proliferation of shoots or roots from the tumor callus. Three additional mutants not caused by Tn*5* contained mutations in the Ti plasmid.

Agrobacterium tumefaciens (Smith and Townsend, Conn.) incites a neoplastic transformation of dicotyledonous plants called crown gall. Oncogenic strains harbor large plasmids of at least 100 megadaltons (Md) which are essential for tumorigenicity (46, 50, 55). Crown gall tumors generally produce either octopine [N^2-(D-1-carboxyethyl)-L-arginine] (35) or nopaline [N^2-(1,3-dicarboxypropyl)-L-arginine] (18, 19), and the genes required for the synthesis (5) and catabolism (5, 22, 29, 30, 38) of these compounds reside on the tumor-inducing (Ti) plasmid. Axenic cloned crown gall tumor lines contain DNA homologous with a region of the Ti plasmid (the T-DNA) (9, 54), which is stably integrated into the plant genome (45). This DNA is believed to encode the "oncogenes." Deletions which extend into the T-DNA (30) and integration of RP4 into the T-DNA (24) result in avirulence. In the plant cell, the T-DNA is transcribed into RNA (17, 20), and at least some of this RNA is polyadenylated (34; A. M. Ledeboer, Ph.D. thesis, University of Leiden, Leiden, The Netherlands, 1978). Furthermore, tumor-derived mRNA encoded by discrete regions of the T-DNA directs the synthesis of specific proteins (molecular weight, 30,000 and 15,000) in a wheat germ cell-free system (34).

Although the T-DNA plays a crucial role in crown gall induction and maintenance, other areas of the Ti plasmid probably encode functions necessary for tumorigenesis. Avirulent mutants resulting from Tn*7* insertion in pTiB6S$_3$:: RP4 (22), and Tn*904* or Tn*1831* insertion in pTiACH5 (28; P. J. J. Hooykaas, Ph.D. thesis, University of Leiden, Leiden, The Netherlands, 1979) have been isolated. In these studies, the mutations were not mapped. Gene products necessary for tumorigenesis might also be encoded by the *Agrobacterium* chromosome or by other cryptic plasmids present in some virulent strains.

In this study, we have utilized the transposon Tn*5* to mutagenize the entire *Agrobacterium* genome of a Ti plasmid-containing strain and have isolated insertion mutants with altered virulence properties or an inability to catabolize octopine. The physical location of the transposon, the mutants' host range, and other phenotypic characteristics have been analyzed in an attempt to gain insight into the genome organization of loci relevant to tumor induction and maintenance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. *Agrobacterium* strains A6NC, a nonclumping derivative of A6 (10), and A114 (formerly NT1), a Ti plasmidless derivative of C58 (50), have been described previously, and mutants are described in the text and Table 1. Although cured of pTiC58, A114 harbors the cryptic plasmid pAtC58, which is larger than 300 Md (6). A722 and A723 were constructed by transforming A114 with pTiA6NC and pTiB$_6$806, respectively, and selecting for growth on agar containing octopine as the sole source of carbon and nitrogen (23). The Ti plasmids pTiA6NC and pTiB$_6$806 are both wide host range octopine-type plasmids with over 87% base sequence homology (12, 16), although pTiA6NC contains a deletion in *Hpa*I-4 (11, 41). *Escherichia coli*

TABLE 1. *List of A. tumefaciens strains*[a]

Strain	Tn5 location	Plasmid	Strain	Tn5 location	Plasmid
A1000	Ti plasmid	pTiA6NC *onc-1*::*Tn5*	A1029	Ti plasmid	$pTiB_6806$ *occ-3*::*Tn5*
A1001	Chromosome	pTiA6NC	A1030	Ti plasmid	$pTiB_6806$ *onc-17*::*Tn5*
A1002	Ti plasmid	pTiA6NC *onc-2*::*Tn5*	A1031	Ti plasmid	$pTiB_6806$ *onc-18*::*Tn5*
A1003	Chromosome	pTiA6NC	A1034	Ti plasmid	$pTiB_6806$ *onc-19*::*Tn5*
A1005	Ti plasmid	pTiA6NC *occ-1*::*Tn5*	A1035	Ti plasmid	$pTiB_6806$ *onc-20*::*Tn5*
A1006	Ti plasmid	pTiA6NC *occ-2*::*Tn5*	A1038	Chromosome	$pTiB_6806$
A1007	Ti plasmid	pTiA6NC *onc-3*::*Tn5*	A1039	Ti plasmid	$pTiB_6806$ *occ-4*::*Tn5*
A1008	Chromosome	pTiA6NC	A1040	Ti plasmid	$pTiB_6806$ *onc-21*::*Tn5*
A1009	Ti plasmid	pTiA6NC *onc-4*::*Tn5*	A1045	Chromosome	$pTiB_6806$
A1010	Ti plasmid	pTiA6NC *onc-5*::*Tn5*	A1056	Ti plasmid	$pTiB_6806$ *occ-5*::*Tn5*
A1011	Chromosome	pTiA6NC	A1057	Ti plasmid	$pTiB_6806$ *occ-6*::*Tn5*
A1012	Ti plasmid	pTiA6NC *onc-6*::*Tn5*	A1058	Ti plasmid	$pTiB_6806$ *occ-7*::*Tn5*
A1016	Ti plasmid	pTiA6NC *onc-8*::*Tn5*	A1059	Chromosome	$pTiB_6806$
A1017	Chromosome	pTiA6NC	A1060	Ti plasmid	$pTiB_6806$ *onc-22*::*Tn5*
A1018	Ti plasmid	$pTiB_6806$ *onc-9*::*Tn5*	A1061	Ti plasmid	$pTiB_6806$ *onc-23*::*Tn5*
A1019	Ti plasmid	$pTiB_6806$ *onc-10*::*Tn5*	A1067	Chromosome	$pTiB_6806$
A1020	Chromosome	$pTiB_6806$	A1068	Chromosome	$pTiB_6806$
A1021	Ti plasmid	$pTiB_6806$ *onc-11*::*Tn5*	A1069	Ti plasmid	$pTiB_6806$ *onc-24*::*Tn5*
A1022	Ti plasmid	$pTiB_6806$ *onc-12*::*Tn5*	A1070	Chromosome	$pTiB_6806$ *onc-25*
A1023	Ti plasmid	$pTiB_6806$ *onc-13*::*Tn5*	A1071	Chromosome	$pTiB_6806$ *onc-29*
A1024	Ti plasmid	$pTiB_6806$ *onc-14*::*Tn5*	A1072	Ti plasmid	$pTiB_6806$ *onc-26*::*Tn5*
A1026	Ti plasmid	$pTiB_6806$ *onc-15*::*Tn5*	A1073	Chromosome	$pTiB_6806$
A1027	Chromosome	$pTiB_6806$ *onc-28*	A1074	Ti plasmid	$pTiB_6806$ *onc-27*::*Tn5*
A1028	Ti plasmid	$pTiB_6806$ *onc-16*::*Tn5*	A1083	Ti plasmid	$pTiB_6806$::*Tn5*
			A1084	Ti plasmid	$pTiB_6806$::*Tn5*
			A1085	Ti plasmid	$pTiB_6806$::*Tn5*

[a] Strains A1000 through A1005 are derived from A6NC. All other strains are derived from A114 containing pAtC58 (6) and either pTiA6NC (A722) or $pTiB_6806$ (A723).

strain 1830, obtained from J. E. Beringer, harbors the conjugative plasmid pJB4J1 (4). This P-type plasmid encodes resistance to gentamycin, chloramphenicol, streptomycin, and spectinomycin. The plasmid also contains the transposon Tn*5* and a MuC^+ prophage.

AB minimal medium (7) was solidified with 1.5% agar (Difco). In some instances, octopine (Sigma) at 500 μg/ml was used as the sole carbon and nitrogen source. Liquid broth consisted of a 1:1 mixture of L (37) and mannitol glutamate broth (LBMG) (3). Octopine differential media (BTB) was used for rapid screening of colonies utilizing octopine (25).

Bacterial matings. The transposon Tn*5*, which encodes kanamycin resistance (1, 2), was introduced into *A. tumefaciens* strains A722, A723, or A6NC by conjugation with 1830 (pJB4J1). Wide host range R factors, such as pJB4J1, which contain an integrated Mu^+ prophage are not stably maintained in *Agrobacterium* (48). Therefore, kanamycin-resistant transconjugants were recovered when Tn*5* transposed from pJB4J1 into the genome of the recipient.

Strain 1830 (pJB4J1) and either A722, A723, or A6NC were grown overnight at 28°C in L and LBMG broth, respectively, to late log phase and mixed in a 1:1 ratio. Sterile filters (0.45-μm pore size, 2.5-cm diameter, Millipore) were placed on nutrient agar (NA, Difco) plates. Approximately 0.1 ml of the mating mixture, or of donor or recipient controls, was spotted onto each filter, and the plates were incubated for 18 h at 28°C. Each filter was then placed in a tube containing 2.0 ml of 0.9% NaCl and blended vigorously in a Vortex mixer; 0.15 ml of the bacterial suspension was spread onto AB containing a final concentration of 100 μg of kanamycin (Sigma) per ml (one plate per filter). Transconjugant colonies appeared (with a frequency of 5×10^{-7} per recipient plated) after 3 days of incubation, whereas control platings of donor or recipient alone were negative. To ensure independent events, only mutants that arose from different filter matings were saved.

Screening for virulence mutants. Kanamycin-resistant transconjugant colonies were transferred to fresh AB plates containing kanamycin. The resulting colonies were inoculated onto *Kalanchoë daigremontiana* leaf wounds made by a sterile toothpick on either side of the midrib. Twenty different isolates plus controls were inoculated per plant. The controls included the virulent *Agrobacterium* recipient used in the mating and strain A114 or an uninoculated wound. After 3 weeks of incubation, the plants were scored for tumor production, and putative mutants were clonally purified and verified by retesting on *Kalanchoë* leaves and stems. Mutants avirulent on *Kalanchoë* were inoculated onto tomato (*Lycopersicum esculentum* var. red cherry), tobacco (*Nicotiana tabacum* var. *xanthi*), sunflower (*Helianthus annuus*), and carrot root slices (*Daucus carota*, local variety). Approximately 5×10^8 bacteria of each mutant, A114, or the parent strain were applied per stem wound. The resulting tumors were compared to control inoculations performed at the same time on other plants. Carrots were washed in soapy water and placed in 5% (vol/vol) bleach (commercial grade) for 30 min. They were then rinsed in sterile water to remove excess bleach

and cut into 1.5-cm sections with a sterile knife, and three to four sections were placed in sterile glass petri dishes containing filter paper (Whatman). After bacteria were spread onto the slices with the edge of a sterile flat toothpick, the slices were incubated at room temperature for 3 weeks.

Tumor line. An axenic tissue culture line was derived from a tumor incited on *N. tabacum* var. white burley by strain A1022. Nutrient broth (Difco) inoculated with tumor tissue was incubated for 3 days and gave no indication of bacterial growth, either by turbidity or by plating on NA.

Isolation of octopine-utilizing revertants. Approximately 10^9 *occ* mutant cells were spread onto agar plates containing 400 μg of octopine (Sigma) per ml as the sole carbon and nitrogen source and incubated at 28°C for 14 days. Octopine-catabolizing revertants were purified and replica plated onto AB containing kanamycin and BTB indicator plates. Reversion frequencies were calculated as the number of octopine-utilizing revertants divided by the total number of bacteria initially plated.

Octopine production. Tumor tissue was excised from tobacco or *Kalanchoë* after 1 month of incubation and assayed for octopine synthesis as described by Otten and Schilperoort (40).

Generation time. The generation time of exponentially growing cells was determined turbidometrically with a Klett-Summerson Photoelectric Colorimeter equipped with a no. 66 filter. Cells were grown to saturation in AB glucose minimal broth and diluted to a turbidity of approximately 10 to 20 Klett units in a final volume of 5 ml of prewarmed AB broth. The cultures were aerated by reciprocal shaking at 28°C and turbidity was measured every hour. Under these conditions 1 Klett unit equals 0.5×10^7 to 1×10^7 bacteria per ml.

Plasmid isolation. Cells were grown in 250 ml of LBMG to late-log phase at 28°C with shaking. Plasmids were isolated by the method of Currier and Nester (13) with slight modifications. Approximately 1.0 g (wet weight) of cells was lysed in 100 ml of lysis buffer, and 0.3 M sodium acetate replaced magnesium chloride and sodium phosphate in the DNA precipitation step. A plasmid screening procedure for 2 to 5 ml of bacterial culture grown in LBMG was used as described by Casse et al. (6). DNA concentrations were determined by the fluorometric method of Lepeq (27).

Gel electrophoresis. Horizontal agarose (Seakem) slab gels were constructed as described previously (11, 41). Electrophoresis was continued until the dye front migrated 20 cm at 1.3 V/cm. Vertical agarose slab gels (0.7%, wt/vol) were used to analyze undigested plasmid preparations. Electrophoresis was performed at 100 V for 5 h with a buffer system of 89 mM Tris, 2.5 mM Na_2EDTA, and 8.9 mM boric acid. Size estimates for undigested plasmids (6, 52) or Ti-plasmid restriction fragments (11) have been reported previously. After electrophoresis and staining in 0.5 μg of ethidium bromide per ml (Sigma), vertical and horizontal gels were illuminated by UV light (model C-61, Ultra-Violet Products, Inc.) and photographed with a MP-4 Land camera (Polaroid) equipped with orange and UV filters (Kodak).

Restriction endonuclease digestions. Restriction endonucleases *HpaI* and *SmaI* were purchased from Bethesda Research Laboratories and Boehringer-Mannheim, respectively, and used according to suppliers' specifications. *KpnI*, obtained from M. Koomey, was used to digest 0.75 μg of DNA in 50 μl of buffer containing 5 mM NaCl, 6 mM Tris-hydrochloride (pH 7.5), 6 mM $MgCl_2$, and 6 mM 2-mercaptoethanol (Sigma) for 3 h at 37°C. Enzyme reactions were terminated by adding 5 μl of stopmix (20% Ficoll, 0.2% sodium dodecyl sulfate, 0.05% bromthymol blue [wt/vol]) and heating at 70°C for 10 min.

DNA filter hybridization. After gel electrophoresis, DNA was transferred to nitrocellulose filters (0.45 μm, Millipore) by the method of Southern (43). Undigested plasmid DNA was depurinated before alkali denaturation by rocking the gel in 0.25 M HCl for 15 min at room temperature (49) to facilitate transfer of high-molecular-weight superhelical DNA molecules to the nitrocellulose filters. The nitrocellulose transfers were used in filter hybridization as described by Thomashow et al. (45). The radioactive probe, ColE1::Tn*5* (obtained from D. Berg) was labeled in vitro to 70×10^6 to 150×10^6 cpm/μg of DNA with [α-^{32}P]dATP, [α-^{32}P]dCTP, and [α-^{32}P]dTTP (New England Nuclear) by the nick translation procedure of Maniatis et al. (32). Autoradiograms were prepared by mounting the filter on cellulose acetate film (Hunt Man Co.) and exposing X-ray film, (X-Omat, Kodak) in the presence of intensifying screens (Dupont Cronex Lightning Plus) for various lengths of time at −70°C.

Symbols. Mutants with altered virulence properties and containing Tn*5* insertions in the Ti plasmid are designated as *onc* followed by the mutant number and Tn*5*, whereas the chromosomal mutants are identified by their strain number. Occ (for octopine catabolism) designates the ability to grow using octopine as the sole carbon and nitrogen source. To indicate specific Ti-plasmid restriction fragments produced by digestion with a particular enzyme, the enzyme name (e.g., *HpaI*) is followed by a hyphen and the restriction fragment number (e.g., *HpaI*-14).

RESULTS

Experimental design. We sought to examine loci relevant to tumor induction and maintenance present in the entire *Agrobacterium* genome by using transposon (Tn*5*) mutagenesis. Mutations due to Tn*5* insertion result from direct gene inactivation or polarity effects and usually result in complete loss of gene function (1). The vehicle used to deliver Tn*5* to *Agrobacterium* was pJB4J1. The plasmid is unstable in *Agrobacterium* (D. Garfinkel and F. White, unpublished data) and also in *Rhizobium* (4). As a result, kanamycin-resistant transconjugants represent Tn*5* insertions in the chromosome, the Ti plasmid, or other cryptic plasmids. The insertions that mutate loci necessary for tumor induction or maintenance or both should fail to incite normal tumors. We therefore inoculated 8,900 kanamycin-resistant transconjugants onto

K. daigremontiana leaf wounds to test for oncogenicity. Subsequently, 40 mutants affected in tumorigenicity were isolated.

The location of Tn*5* insertions was determined with respect to cleavage sites for restriction endonucleases in the Ti plasmid and in Tn*5*. Fractionation of the digested plasmids by agarose gel electrophoresis permitted the assignment of the Tn*5* insertion to specific restriction fragments of the Ti plasmid. If the Ti plasmid did not contain Tn*5*, it could be inserted in either the *Agrobacterium* chromosome or a cryptic plasmid pAtC58 (6). The strategy used to locate these insertions involved the isolation of total plasmid DNA from the mutant strains and fractionation of undigested plasmid DNA by agarose gel electrophoresis. The Ti plasmid pAtC58 and linear plasmid and chromosomal DNA fragments were transferred to nitrocellulose filter paper and hybridized with a ^{32}P-labeled Tn*5* probe. Hybridization with only the linear fragments indicated that Tn*5* was present in the chromosome and not in pAtC58. This analysis also confirmed that Tn*5* was not present in the Ti plasmid.

The relationship between a mutant phenotype associated with Tn*5* insertion and the presence of the Ti plasmid was examined for avirulent mutants which did not contain Ti plasmid-borne Tn*5* insertions and for mutants which contained T-DNA insertions. Ti plasmid from each mutant was transformed into a Ti plasmid-less strain (A114), selecting for Occ$^+$ transformants. The transformants which contained Ti plasmids from mutants with Tn*5* in the chromosome or pAtC58 were screened for tumorigenicity and kanamycin resistance. A virulent kanamycin-sensitive transformant indicated that the parental Ti-plasmid did not contain an *onc* mutation, and suggested that Tn*5*-mutated chromosomal or pAtC58-encoded genes affecting virulence. The transformants containing T-DNA insertions were analyzed for virulence, for kanamycin resistance, and for the location of the insertion to determine whether the Ti plasmid was responsible for the Onc$^-$ phenotype.

Restriction map location of Ti-plasmid insertions affecting virulence. Of the 8,900 kanamycin-resistant transconjugants analyzed for oncogenicity, 37 independently isolated mutants had Tn*5* insertions in the *Agrobacterium* genome that affected virulence. Of the 37 mutants, 25 contained Tn*5* insertions in pTiA6NC (7 insertions) or pTiB$_6$806 (18 insertions), as initially shown by *Kpn*I restriction enzyme digest patterns of the purified Ti plasmid (data for 5 of the 25 mutants are shown in Fig. 1). To prevent ambiguities in locating Tn*5* insertions due to the complexity of the Ti-plasmid restriction digest patterns (i.e., fragments of similar molecular weight such as *Sma*I-10a, -10b, and -10c), the insertions were further mapped with *Hpa*I and *Sma*I. Tn*5* (3.5 Md) contains two *Hpa*I sites, located in the inverted repeats approximately 0.2 Md from the end of the element, one *Sma*I site located approximately in the center of the element, and no *Kpn*I sites (26). The locations of the restriction sites for *Kpn*I, *Hpa*I, and *Sma*I are also known for pTiB$_6$806 and pTiA6NC (11, 41; G. Ooms, personal communication).

For example, digesting pTiB$_6$806 *onc*-27::Tn*5* with *Kpn*I increased the molecular weight of *Kpn*I-5 by the molecular weight of Tn*5* (Fig. 1, *Kpn*I, lane a). Digesting with *Hpa*I generated an internal Tn*5* fragment which co-migrated with pTiB$_6$806 or pTiA6NC *Hpa*I-11, as well as two Tn*5*–*Hpa*I-12 junction fragments of 1.5 and 2 Md (*Hpa*I, lane a). Digesting with *Sma*I generated two Tn*5*–*Sma*I-1 junction fragments of 8 and 10 Md in place of the original fragment due to Tn*5* insertion (*Sma*I, lane a). The locations of three other Tn*5* insertions in the Ti plasmid are 370summarized as follows: pTiB$_6$806 *onc*-26:: *Tn5* summarized as follows: pTiB$_6$806 *onc*-26:: *Tn5* (lane b), *Hpa*I-14, *Kpn*I-10, *Sma*I-10c; pTiB$_6$806 *onc*-11::*Tn5* (lane d), *Hpa*I-6, *Kpn*I-5, *Sma*I-8; and pTiB$_6$806 *onc*-22::Tn5 (lane e), *Hpa*I-3, *Kpn*I-2, *Sma*I-8 (Fig. 1).

Strains A1027, A1070, and A1071 contained mutations in pTiB$_6$806 which affect tumorigenesis (Table 2), but were not caused by Tn*5*. In A1070 (pTiB$_6$806 *onc*-25) an insertion of approximately 1.0 Md was present in *Hpa*I-14 (Fig. 1, lane c), but it did not have any of the expected restriction sites of Tn*5*. Most striking was the absence of the Tn*5* internal *Hpa*I fragment of 3.2 Md (*Hpa*I, lane c), because this fragment contains the kanamycin resistance locus (26). Since A1070 was kanamycin resistant, a copy of Tn*5* must be present somewhere in the genome, presumably in the chromosome or pAtC58. The origin of the 1.0-Md insert present in *Hpa*I-14 remains unexplained. The Ti-plasmids from A1027 (pTiB$_6$806 *onc*-27) and A1071 (pTiB$_6$806 *onc*-28) were digested with *Kpn*I and analyzed by agarose gel electrophoresis. The restriction patterns of the Ti plasmids (pTiB$_6$806 *onc*-27 and *onc*-28) were indistinguishable from the parental pTiB$_6$806 plasmid (data not shown). In addition, only chromosomal DNA from A1027 and A1071 hybridized with a ^{32}P-labeled Tn*5* probe (see Fig. 5, lanes b and f, respectively). These results indicate that Tn*5* is present in the chromosome and not in the Ti plasmid. The Ti plasmids from A1027 and A1071 were transformed into A114, and tested for virulence on

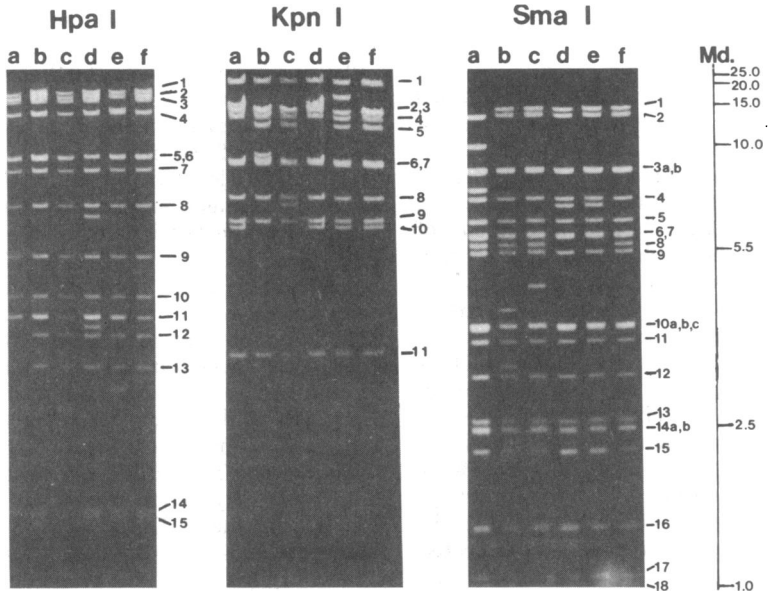

FIG. 1. *Agarose gel electrophoresis of HpaI, KpnI, and SmaI digests of representative pTiB$_6$806 plasmids containing insertions; (a) pTiB$_6$806 onc-27::Tn5; (b) pTiB$_6$806 onc-26::Tn5; (c) pTiB$_6$806 onc-25; (d) pTiB$_6$806 onc-11::Tn5; (e) pTiB$_6$806 onc-22::Tn5; (f) wild-type pTiB$_6$806 plasmid. Restriction digest fragments are numbered to the right of each gel. Molecular size estimates are reported in megadaltons.*

Kalanchoë and for kanamycin resistance. The transformants were kanamycin sensitive, but were avirulent on *Kalanchoë* leaves, indicating that the Ti plasmids from A1027 and A1071 were mutated.

The insertion map shows a clustering of the 26 insertions affecting tumorigenesis (Fig. 2, Onc$^-$ phenotype). Twenty-three mutants contained *Tn5* insertions in a 20-Md region bordered by *Hpa*I-9 and the right end of *Sma*I-8. Two Tn*5* insertions and the 1.0-Md insert present in A1070 were located in *Hpa*I-14. DNA homologous to *Hpa*I-14 has been found in all tumor lines examined (36, 45, 54), and in many Ti plasmids (8, 15, 21). None of the 26 Ti-plasmid insertions was located in a region (*Sma*I-3b through *Sma*I-4) not essential for virulence (30).

Tumor morphology mutants. Twenty-one of twenty-five Tn*5*-induced mutants, as well as A1027 and A1071, were avirulent on all plants tested. Several mutants were isolated which induced tumors with altered morphologies (Table 2). Mutants A1028 (pTiB$_6$806 *onc*-16::*Tn5*) and A1034 (pTiB$_6$806 *onc*-19::*Tn5*) mapped in *Hpa*I-6 (Fig. 2). These strains induced normal-looking galls when inoculated onto sunflower stems, carrot root slices, or tomato stems. On *Kalanchoë* stems and leaves and tobacco stems, their phenotype was morphologically different from the tumor produced by the virulent isogenic parent strain A723. The parent strain induced an undifferentiated tumor from the wound inoculation site on *Kalanchoë* leaves and tobacco stems, and roots proliferated from the tumor periphery on *Kalanchoë* stems. Mutants A1028 and A1034 repeatedly produced tumors with roots proliferating from small calluses on tobacco stems and *Kalanchoë* leaves. On *Kalanchoë* stems, roots proliferated from the entire callus. Tumor calluses from *Kalanchoë* stems and leaves synthesized octopine (40).

The T-DNA mutants (Table 2) contained insertions in the 1.4-Md *Hpa*I-14 fragment. Two of the mutants, A1022 (pTiB$_6$806 *onc*-12::*Tn5*) and A1072 (pTiB$_6$806 *onc*-26::*Tn5*), produced the same altered tumor morphology on the host plant. This phenotype was characterized by a lack of root proliferation on *Kalanchoë* stems and necrotic callus formation on both *Kalanchoë* stems and leaves (Table 2, Fig. 3). On tobacco stems, shoots proliferated from an unorganized callus. This type of tumor morphology was not observed with strains harboring the parent pTiB$_6$806 plasmid. The sites of the Tn*5* insertions were not identical in A1022 and A1072, since the respective insertions generated different *Hpa*I restriction fragments containing the Tn*5*-Ti plasmid junction (data not shown).

A tobacco tumor induced by strain A1022 was put into axenic culture, and the proliferation of shoots continued in the absence of the inciting organism. The undifferentiated callus was phytohormone independent and produced octopine (31). Shoots did not produce detectable levels of

octopine, and stem explants failed to grow in culture unless phytohormones (an auxin plus a cytokinin) were added to the media (D. Garfinkel and S. Dellaporta, unpublished data).

Strain 1070 (pTiB$_6$806 *onc*-25) contained a 1.0-Md insertion in *Hpa*I-14 and incited tumors that had a unique appearance. Although A1070 was avirulent on *Kalanchoë* leaves, massive amounts of roots proliferated from a small callus when this mutant was inoculated onto *Kalanchoë* stems (Fig. 4, Table 2). On carrot root slices and tobacco stems, roots developed from the callus. Roots were not observed when the isogenic parent A723 was tested on tobacco or carrot root

TABLE 2. *Virulence of A. tumefaciens strains harboring mutated Ti plasmids*

Strain	Position of insertion			Virulence						Octopine[a] production
	*Hpa*I	*Kpn*I	*Sma*I	*Kalanchoë*		Carrot	Sunflower	Tomato	Tobacco	
				Stems	Leaves					
Controls										
A114	–	–	–	–[b]	–	–	–	–	–	Negative
A722	–	–	–	+[c]	+[d]	+[d]	+[d]	+[d]	+[d]	Positive
A723	–	–	–	+[c]	+[d]	+[d]	+[d]	+[d]	+[d]	Positive
A6NC	–	–	–	+[c]	+[d]	+[d]	+[d]	+[d]	+[d]	Positive
Avirulent										
A1007(*onc-3*)	9	11	1	–	–	–	–	–	NT[e]	NT
A1016(*onc-8*)										
A1030(*onc-17*)										
A1069(*onc-24*)	16	5	1	–	–	–	–	–	NT	NT
A1019(*onc-10*)	15	5	1	–	–	–	–	–	NT	NT
A1023(*onc-13*)										
A1031(*onc-18*)										
A1035(*onc-20*)										
A1040(*onc-21*)										
A1000(*onc-1*)	12	5	1	–	–	–	–	–	NT	NT
A1009(*onc-4*)										
A1012(*onc-6*)										
A1018(*onc-9*)										
A1024(*onc-14*)										
A1074(*onc-27*)										
A1010(*onc-5*)	6	5	1	–	–	–	–	–	NT	NT
A1061(*onc-23*)										
A1021(*onc-11*)	6	5	8	–	–	–	–	–	NT	NT
A1002(*onc-2*)	3	2	8	–	–	–	–	–	NT	NT
A1026(*onc-15*)										
A1060(*onc-22*)										
A1027(*onc-28*)	ND[e]	ND	ND	–	–	–	–	–	NT	NT
A1071(*onc-29*)	ND	ND	ND							
Tumor morphology										
A1028(*onc-16*)	7	2	8	+[f]	+[g]	+[d]	+[d]	+/–[i]	+[f]	Positive
A1034(*onc-19*)	6	5	1	+[f]	+[g]	+[d]	+[d]	+/–	+[f]	Positive
A1022(*onc-12*)	14	10	10c	+[h]	+[h]	+/–	+[d]	+/–	+[j]	Positive[k]
A1070(*onc-25*)	14	10	10c	+[g]	–	+[g]	+[d]	+/–	+[g]	Positive
A1072(*onc-26*)	14	10	10c	+	+	+/–	+/–	+/–	+	Positive[k]

[a] Assayed by the method of Otten and Schilperoort (40).
[b] Avirulent.
[c] Unorganized callus overgrowth with roots proliferating from the callus periphery.
[d] Unorganized callus overgrowth.
[e] NT, Not tested; ND, not determined.
[f] Abnormal callus with roots proliferating from the entire callus.
[g] Unorganized callus with roots proliferating from the callus overgrowth.
[h] Necrotic callus overgrowth.
[i] Unorganized callus, but smaller in size than tumors induced by the parent strain.
[j] Unorganized callus with shoots proliferating from the entire callus.
[k] Unorganized callus produced octopine, but shoots did not produce detectable levels of octopine.

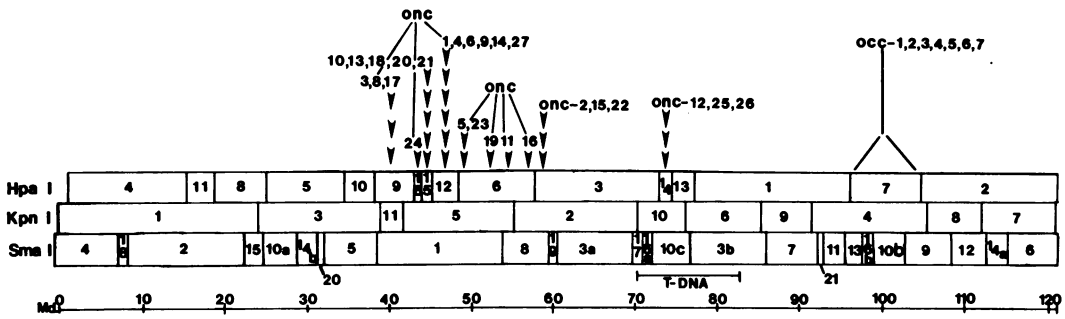

FIG. 2. *Restriction map of pTiB$_6$806 containing insertions affecting virulence (onc) and octopine utilization (occ). The T-DNA is represented by a bar. Molecular size estimates are reported in megadaltons.*

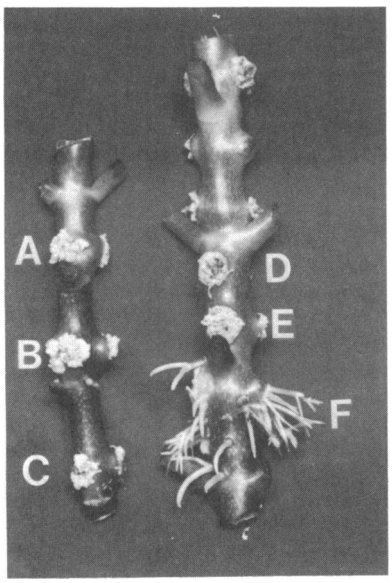

FIG. 3. *Kalanchoë stem inoculated with strain A1022 (A to E), and the isogenic parent A723 (F) after 1 month of incubation. The leaves were removed and the stem was excised from the plant before photography.*

slices. Undifferentiated tissue from *Kalanchoë* or tobacco tumors incited by strains A1022, A1070, or A1072 produced octopine (40). The plasmids containing T-DNA insertions were transformed into strain A114, and transformants retained all characteristics of the original mutants. As predicted, transformants containing pTiB$_6$806 *onc*12::*Tn5* or pTiB$_6$806 *onc-26*::*Tn5* were resistant to kanamycin, whereas transformants containing pTiB$_6$806 *onc-25* were kanamycin sensitive.

Tn*5* insertions affecting virulence which do not reside on the Ti plasmid. We isolated 12 avirulent mutants containing Tn*5* insertions which could not be located on the Ti plasmid by restriction endonuclease analysis. The Ti plasmids from these mutants conferred virulence when transformed into strain A114, and the transformants were kanamycin sensitive. Presumably, Tn*5* caused a mutation in genes required for virulence which are not encoded by the Ti plasmid. For strain A6NC these genes probably reside on the *Agrobacterium* chromosome because pTiA6NC is the only plasmid which could be detected in this strain by using techniques which have demonstrated plasmids over 200 Md in *A. tumefaciens* (6) and *Agrobacterium rhizogenes* (52). For strains A722 and A723, these genes could be encoded by the chromosome or pAtC58. The plasmid pAtC58 could be easily separated from pTiB$_6$806 or pTiA6NC after agarose gel electrophoresis of undigested plasmid preparations. A nitrocellulose filter transfer was made from the gel containing total plasmid DNA isolated from seven mutants and 2 positive controls (Fig. 5, I), and hybridized with a radiolabeled Tn*5* probe. Figure 5 II is an autoradiogram made from the nitrocellulose filter after hybridization. If Tn*5* had been present in the Ti plasmid or pAtC58, these plasmids would have hybridized with the Tn*5* probe. However, only the positive controls pJB4J1 and pTiB$_6$806 *onc-26*::*Tn5* hybridized with the Tn*5* probe. In 12 mutants analyzed in this manner, ^{32}P-labeled Tn*5* hybridized with the linear DNA fragments, indicating that Tn*5* inserted into the *Agrobacterium* chromosome. Extended autoradiography did not alter this result.

Seven of the twelve chromosomal mutants had an altered host range (Table 3). Two mutants, A1008 and A1067, incited galls on sunflower and tomato plants, although they were avirulent on *Kalanchoë*. The remaining host range mutants were virulent only on sunflower and were avirulent on *Kalanchoë* and tomato. Five chromosomal mutants were avirulent on all plants tested. None of the chromosomal mutants had prominent differences in colony morphology

when grown on NA or AB glucose minimal agar. Parent strains A722, A723, and A6NC had an average generation time of 3.5 h (three experiments) when grown at 28°C in AB glucose liquid medium. All mutants had comparable generation times (± 20%) when grown under the same conditions (details of experimental procedure in Materials and Methods).

Map location and phenotype of Tn*5*-induced octopine catabolic mutants. Seven mutants (frequency of 0.1%) failed to develop strong yellow-colored colonies indicative of octopine catabolism when plated on octopine bromothymol blue indicator agar (25). Instead, clear watery colonies similar to Ti plasmid-less strain A114 were observed. These mutants contained Tn*5* plasmid-borne insertions in restriction fragments *Hpa*I-7 or *Kpn*I-4 (Fig. 2, Occ^- phenotype), and their map position independently confirmed the location of the *occ genes* previously mapped from deletion analyses (30). The seven mutants also failed to grow on solid media with octopine as the sole source of carbon and nitrogen. Octopine-utilizing revertants were obtained with a frequency of 5×10^{-8}. All were kanamycin sensitive, indicating that the Tn*5* excision repaired gene function and that Tn*5* was lost from the cell (1). All mutants were virulent when tested on *Kalanchoë*.

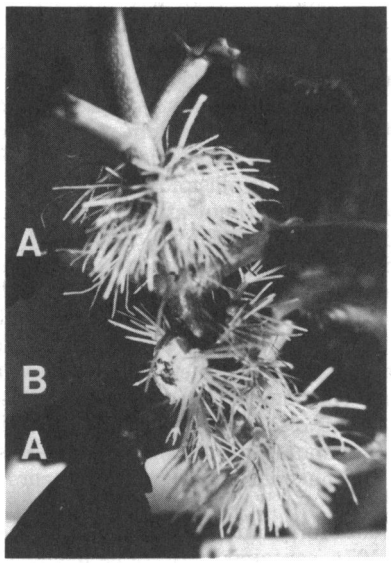

FIG. 4. *Kalanchoë stems inoculated with strain A1070 (A) and the isogenic parent A723 (B) after 1 month of incubation.*

Stability and specificity of Tn*5* insertion. Tn*5* was examined for preferential insertion between the chromosome and the plasmid. Kanamycin-resistant virulent transconjugants were picked at random and analyzed as described previously for the presence of Tn*5* in the Ti plasmid, pAtC58, or the *Agrobacterium* chromosome. Three of the forty transconjugants screened (7.5%) contained Tn*5* insertions in the Ti plasmid. Two strains (A1084 and A1085) contained Tn*5* insertions in *Kpn*I-8, and one strain (A1083) contained an insertion in *Kpn*I-9. Based on the relative target sizes of $pTiB_6806$ (120 Md) and the *Agrobacterium* chromosome, these re-

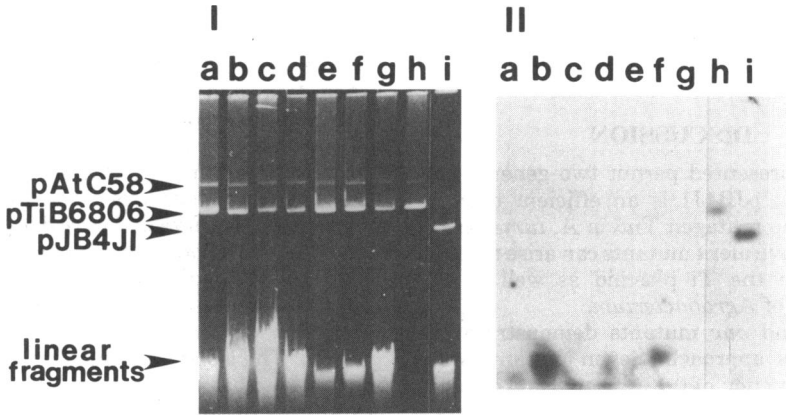

FIG. 5. *(I) Agarose gel electrophoresis of undigested plasmids from strains; (a) A1020; (b) A1027; (c) A1038; (d) A1045; (e) A1068; (f) A1071; (g) A1073; (h) $pTiB_6806$ onc-26::Tn5; (i) pJB4J1. The molecular size estimate for pAtC58 is over 300 Md (6); $pTiB_6806$ is 120 Md (11), and pJB4J1 is approximately 60 Md (D. Garfinkel, unpublished data). (II) Hybridization of Tn5 insert present in strains; (a) A1020; (b) A1027; (c) A1038; (d) A1045; (e) A1068; (f) A1071; (g) A1073; (h) $pTiB_6806$onc-26::Tn5; (i) pJB4J1. The DNA in the agarose gel (Fig. 5 I) was transferred to a nitrocellulose filter (43, 49), hybridized with a ^{32}P-labeled ColE1::Tn5 probe (45), and autoradiographed for various lengths of time. If only the linear fragments hybridized with ColE1::Tn5, the transposon is present in the Agrobacterium chromosome*

TABLE 3. *Virulence of chromosomal Tn5 insertion mutants*

Strain	Virulence			
	Kalanchoë		Sunflower	Tomato
	Stem	Leaves		
Controls				
A114	−[a]	−	−	−
A722	+[b]	+[c]	+[c]	+[c]
A723	+[b]	+[c]	+[c]	+[c]
A6NC	+[b]	+[c]	+[c]	+[c]
Avirulent				
A1001, A1011, A1020, A1038, A1045	−	−	−	−
Host range				
A1003, A1017, A1059, A1068, A1073	−	−	+[c]	−
A1008, A1067	−	−	+[c]	+[c]

[a] Avirulent.
[b] Unorganized callus overgrowth with roots proliferating from the callus periphery.
[c] Unorganized callus overgrowth.

sults suggest that Tn*5* demonstrates little preference for plasmid versus chromosomal insertion.

All mutants were stably kanamycin resistant and retained their original phenotype through at least one subculture in the absence of kanamycin. To further examine the stability of a specific Onc$^-$ phenotype, strain A1022 was grown in the absence of kanamycin, and 1,800 colonies were individually inoculated on *Kalanchoë* leaves. All subclones incited tumors that were identical to the original tumor morphology in the absence of selective pressure to retain Tn*5*.

DISCUSSION

The data presented permit two general conclusions: first, pJB4J1 is an efficient delivery vehicle for the mutagen Tn*5* in *A. tumefaciens* and, second, avirulent mutants can arise through alterations in the Ti plasmid as well as the chromosome of *Agrobacterium*.

The *occ* and *onc* mutants demonstrate the utility of this approach. Seven mutants were isolated (frequency of 0.1%) from 8,900 colonies screened for the Occ$^-$ phenotype. This frequency compares favorably with that obtained by Beringer et al. (4) using Tn*5* (and the pJB4J1 delivery system) to isolate different catabolic or auxotrophic mutants in *Rhizobium*. The *occ* mutants contained a Tn*5* element in a specific region of pTiB$_6$806 or pTiA6NC (*Hpa*I-7 and *Kpn*I-4). Their location confirms the results of Koekman et al. (30) who, using a completely different approach, isolated deleted Ti plasmids which had lost the *occ* locus. All seven mutants reverted to Occ$^+$ with the concomitant loss of kanamycin resistance at a frequency of 5×10^{-8}. These results indicate that the Tn*5* excision repaired gene function and the transposon was subsequently lost from the cell, similar to precise excision of Tn*5* in *Escherichia coli* (1). The Tn*5* insertions found in 25 *onc* mutants were located in a specific region of the Ti plasmid (Fig. 2). The clustering was not due to a high specificity for insertion in this region because Tn*5* insertions present in virulent strains (A1083, A1084, and A1085) picked at random were not located in the *onc* region. These insertions are located in an area of the Ti plasmid (*Kpn*I-8 or -9) which can be deleted without affecting tumorigenesis (30). If Tn*5* was not the mutagen, *onc* mutants would have been isolated containing insertions dispersed throughout much of the Ti-plasmid such as in *Kpn*I-8 or 9. In addition, both the *occ* and *onc* Tn*5*-induced mutants appeared to contain a complete Tn*5* element as shown by restriction enzyme analysis. The characteristics of the *occ* and *onc* mutants suggest that: (i) Tn*5* causes the mutation, (ii) the mutants are stable, and (iii) the entire transposon appears to be present.

Three of forty mutants isolated, A1027, A1070, and A1071, contained mutations in the Ti plasmid that were not caused by Tn*5*. The Ti-plasmid transformants were all kanamycin sensitive but retained the parental strain's Onc$^-$ phenotype. The insertion present in strain A1070 contained a 1.0-Md insertion in the T-DNA (*Hpa*I-14). This insertion is not homologous with pJB4J1 or ColE1::*Tn5* (D. Garfinkel, unpublished data) and probably represents an *Agrobacterium* insertion sequence (IS). The basis for the mutations in strains A1027 and A1071 has not been determined. These mutants may have resulted from endogenous IS transposition activity in *Agrobacterium* and were detected because almost 10,000 colonies were tested for virulence. It is also possible that pJB4J1 is involved in the generation of these mutants because all colonies tested for virulence transiently harbored this plasmid. The isolation of such mutants makes it imperative that a Ti-plasmid be analyzed by several restriction endonucleases, as well as by plasmid transfer before conclusions are drawn.

Twenty-one avirulent mutants contained Tn*5* insertions located in a specific 20-Md region of the Ti plasmid (Fig. 2, *Sma*I-1 and *Sma*I-8). Eleven of these insertions occurred in two relatively small contiguous restriction fragments, *Hpa*I-12 and *Hpa*I-15 (combined molecular

weight of 4.6×10^6). A preliminary report by Van Montagu and Schell indicates that the same region of a closely related Ti plasmid, pTiACH5, is essential for virulence (47). This region of the Ti plasmid is highly conserved among many Ti plasmids which share low overall sequence homology with $pTiB_6806$ and pTiA6NC (16, 21, 53). These data suggest that genes encoded by this region of the Ti plasmid play an indispensible role in tumorigenesis. The functions altered in these mutants are presently unknown, but could involve plasmid-dependent adherence to plant cells (33, 42, 51) or transmission and stable integration of Ti-plasmid sequences into the plant genome.

The possible relationship between Ti-plasmid conjugation activity and the transmission of the Ti plasmid to susceptible plant cells has not been resolved. Tempé et al. (44) reported that the temperature optima for oncogenicity and Ti-plasmid conjugation are similar, indirectly suggesting that common functions are required for both processes. Ti-plasmid transfer and virulence functions are not completely homologous, since virulent transfer-deficient mutants have been isolated (30). Since the approach used in this study for isolating avirulent mutants does not depend upon Ti-plasmid transfer, avirulent transfer-deficient mutants were not selected against. The analysis of avirulent mutants generated by this approach should help clarify the relationship between bacterial conjugation and oncogenicity.

Crown gall induction requires actively metabolizing *Agrobacterium*, and mutations which impair growth of this organism are less infective (31). However, the avirulent and host range chromosomol mutants grew prototrophically, with generation times comparable to their parent strains. This result indicates that the genes affected by Tn*5* insertions in these mutants are required for virulence on at least *Kalanchoë*. These gene products are either involved with growth under conditions not investigated in this study or are involved with other functions required for tumorigenesis. Such functions might include genes which code for the synthesis of siderophores (39), adherence determinants (51), or functions necessary to overcome plant defense mechanisms (14).

Mutants A1028 and A1034 share the phenotype of inducing aberrant tumors on *Kalanchoë* stem, leaves, and tobacco stems. However, the Tn*5* insertions mapped in *Hpa*I-6, a region of the Ti plasmid that has not been detected in crown gall tissue (9, 36). The unusual tumor morphology could be mediated by continued activity of *Agrobacterium* in the plant wound and developing tumor and may not be a property of the transformed plant cells. This possibility will be tested by removing the bacteria after the induction phase of tumorigenesis and examining the morphology of the resulting axenic tumor in culture. Alternatively, *Hpa*I-6 may be initially transferred to plant cells during tumorigenesis and then lost during later stages of tumor development or during establishment of the axenic cloned cell lines.

Mutants A1022, A1070, and A1072 contained insertions in a region of the T-DNA (Fig. 2, *Sma*I-10c, -16a, and -17) that is detected in all transformed cell lines examined (36, 45, 54). This conserved or common T-DNA is homologous with DNA found in many Ti plasmids that share low overall sequence homology (8, 15, 16, 21). The conserved T-DNA sequences are transcriptionally active in three octopine-type tumors (20), and RP4 cointegration into DNA homologous with *Sma*I-10c (Fig. 2) results in loss of virulence (24). These data suggest that genes encoded in this region of the T-DNA are concerned with tumor induction or maintenance or both and probably function in the plant cell. The insertions present in *Hpa*I-14 therefore mutate genes which could also function in the plant cell during or after transformation. However, the existence of Tn*5* sequences in the plant genome remains to be shown.

In general, A1022 and A1072 incited unusual tumors which proliferate shoots or suppress normal root formation, whereas A1070 incited tumors which proliferate roots (Table 2, Fig. 3 and 4). Tobacco tumors induced by A1022 were placed into axenic tissue culture, and the aberrant shoot formation continued. This observation suggests that plant cells, and not the continued activity of A1022 in the developing gall, are responsible for the morphological change. Shoot explants did not grow in culture in the absence of phytohormones (D. Garfinkel and S. Dellaporta, unpublished data) and did not synthesize detectable levels of octopine. These results suggest that the shoots are comprised of untransformed cells. Possibly, the mutated T-DNA of A1022 functions aberrantly in transformed plant cells, causing neighboring untransformed cells to differentiate into shoots. Further insight into the basis for shoot differentiation should be gained by obtaining single cell clones of the A1022 tumor line and examining their ability to cause formation of shoots by normal callus.

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ADDENDUM IN PROOF

Holsters et al. (Plasmid 3:212-230, 1980) have recently identified a region of a nopaline Ti plasmid (pTiC58) responsible for tumorigenesis.

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