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Characterization of Tn*904* Insertions in Octopine Ti Plasmid Mutants of *Agrobacterium tumefaciens*

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Seven Tn*904* insertion mutants of pTi Ach5 affecting *Agrobacterium tumefaciens* virulence were studied. The mutant character was shown to be plasmid borne. Four of these mutants were avirulent and carried an insertion in restriction endonuclease *Hpa*I fragment 12, a 3.3-megadalton fragment, which therefore appears to be a Ti plasmid region essential for virulence. Two mutants were attenuated in virulence. The inserts mapped close to *Hpa*I fragment 12. One mutant giving rise to small tumors with excessive adventitious root formation on *Kalanchoe daigremontiana* carried an insertion in the right side of the common sequence in the deoxyribonucleic acid of the Ti plasmid detected in crown gall tumors. The insertion behavior of Tn*904* was studied by analyzing 11 independently isolated and randomly chosen mutants. The Tn*904* inserts did not affect oncogenicity, tumor morphology, bacterial transfer functions, octopine catabolism functions, or vital parts of the Ti plasmid, such as the origin of replication. Most of the Tn*904* inserts were concentrated in a small part of the map. The size of additional deoxyribonucleic acid as a result of Tn*904* inserts varied between 5 and 15 megadaltons. In two cases a Ti plasmid was found with two Tn*904* insertions at different positions.

The plant disease crown gall is the result of an infection of a wounded dicotyledonous plant with *Agrobacterium tumefaciens* (40). *Agrobacterium* strains are only virulent when they carry a large plasmid called a tumor-inducing (Ti) plasmid (42-44). Part of the Ti plasmid (the T-DNA) is present in all crown gall cells and is stably maintained (6, 41). The T-DNA is also expressed as polyadenylate-containing RNA (14, 17; A. M. Ledeboer, Ph.D. thesis, Rijksuniversiteit te Leiden, Leiden, The Netherlands, 1978). Three types of Ti plasmids have been recognized in wild-type *Agrobacterium* strains, i.e., octopine, nopaline, and "null type" Ti plasmids. Analysis of these three types of Ti plasmids revealed that they differ on the basis of DNA homology (10, 13, 20, 30) and in several genetic traits, e.g., morphology of the tumors they incite, synthesis by tumors of unusual amino acid derivatives belonging to the octopine or nopaline type of compounds and specific utilization of these compounds by the bacterium, exclusion of phage AP1, sensitivity to agrocin K84, and genes that code for a conjugation system of the Ti plasmid and for incompatibility (for review, see references 1, 5, 24, 34, and 35). To estimate the positions of certain plasmid-encoded genetic markers relative to a restriction map of an octopine Ti plasmid, several Ti plasmid deletion mutants were studied (29). Transposon mutagenesis is another approach to study the genetic organization of Ti plasmids. This has been described by Hernalsteens et al. (21) and Klapwijk et al. (27). In their studies, however, no correlation was made between the insertion position on the physical map of the octopine Ti plasmid and the mutant character. As Tn*904* used by Klapwijk et al. (27) codes for resistance against streptomycin, it is of interest not only to study its use as a site-specific mutagen but also to see whether the antibiotic resistance is expressed in tumor cells when Tn*904* has inserted in T-DNA. In this paper we present mapping data of mutants from a previous study (27) that were shown to be affected in plasmid-borne functions. Moreover, the Tn*904* insertion positions of 11 randomly chosen, independently isolated Ti plasmid mutants were determined in order to see whether there is any preferential insertion site in the octopine Ti plasmid for this transposon.

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MATERIALS AND METHODS

Bacterial strains. Wild-type *A. tumefaciens* strain Ach5, designated LBA4001 in our collection, was obtained from R. H. Hamilton (Pennsylvania State University). The numbers given to the analyzed mutant strains and the numbers given to the analyzed mutant Ti plasmids are listed in Table 2. The plasmid harbored by LBA4001 is pTi Ach5. The Tn*904* insertion mutant strains were isolated by Klapwijk et al. (27). The isolation and characterization of Ti plasmid deletion mutant strains used for mapping *Kpn*I and *Xba*I restriction enzyme recognition sites have been de-

scribed by Koekman et al. (29).

Enzymes. Restriction endonuclease *Sma*I was isolated according to an unpublished method of C. Mulder, and *Kpn*I was purified by following the procedure of Greene et al. (16). Restriction endonucleases *Hpa*I and *Xba*I were generous gifts from, respectively, M. Lupker (Sylvius Laboratory, Leiden) and R. A. Flavell (Jan Swammerdam Institute, Amsterdam).

Plasmid isolation, restriction endonuclease treatment, and gel electrophoresis. Plasmid isolations were performed by following a slightly modified procedure obtained from M.-D. Chilton that was essentially based on a method developed by Hansen and Olsen (18). Cells were grown in a 500-ml culture of TY medium (5 g of tryptone and 3 g of yeast extract per liter) to an optical density at 600 nm of 0.8 to 1.2 under aerobic conditions (2-liter flask, 29°C, 250 rpm; Lab-Therm lab shaker, A. Kühner, AG, Basel). The cells were harvested, lysed, and alkali-treated according to the procedure of Currier and Nester (9) without shearing the lysate. The lysate was brought up to 1 M NaCl and refrigerated (4°C) for 4 h or overnight, as convenient. The salt-precipitated chromosome-membrane complexes were pelleted by centrifugation (4°C, 15 min, 5,000 rpm) in a Beckman JA10 rotor. The supernatant was brought up to 10% 6,000-molecular-weight polyethylene glycol (E. Merck A. G., Darmstadt, West Germany), refrigerated overnight (4°C), and centrifuged at 7,000 rpm (4°C, 20 min) in a Beckman JA10 rotor. The concentrated plasmid DNA was carefully suspended in 50 mM Tris–20 mM EDTA, pH 8.0, and cesium chloride-ethidium bromide gradients were prepared as described previously (29). The ethidium bromide was removed from the plasmid preparations by at least three extractions with isopentyl alcohol saturated with 20× SSC. The DNA was further purified by overnight dialysis (4°C) against 3% NaCl and one or two extractions with phenol (Merck) saturated with 3% NaCl. After precipitation with 2 volumes of ethanol, the pelleted DNA (20 min, 4°C, 10,000 rpm in a Sorvall centrifuge) was washed with 70% ethanol in water and with 96% ethanol. The pellet was dried by blowing a gentle stream of nitrogen through the tube and dissolved in 0.2 ml of water.

Restriction enzyme fragmentation and gel electrophoresis were performed as described previously (29). Restriction enzyme treatment of plasmid DNA with *Kpn*I was carried out at 37°C in a buffer containing 6.7 mM Tris-hydrochloride, pH 7.4, (37°C), 6.7 mM $MgCl_2$, 50 mM NaCl, and 6.7 mM β-mercaptoethanol; *Xba*I digests were made in the same buffer supplemented with an additional 100 mM NaCl. Double digestions were performed in which combinations of *Hpa*I, *Sma*I, *Kpn*I, and *Xba*I were used. These were carried out sequentially by ethanol precipitating enzyme-treated plasmid DNA and further incubation in an appropriate buffer with a second enzyme or, if convenient, by adding salt to an incubation mixture without inactivation of the first enzyme and the addition of a second enzyme, after which the incubation was continued.

Virulence tests. Bacteria were grown in TY medium for 1.5 days and from these late-log-phase cultures were applied directly to wounded plants. *Kalanchoe daigremontiana* tumors were induced on the stems of plants ca. 20 cm high. With a rectangular punch three small holes were made through the stem in three different internodes, and about 4 h later the wounds were filled with a bacterial suspension. Tumors on tomato (*Lycopersicon esulentum*) or petunia (*Petunia hybrida*) plants 3 to 6 weeks old were induced by making a wound through the stem with a Pasteur pipette. The wounds were immediately infected. The petunia plants were kindly provided by F. Bianchi (Amsterdam). D-Lysopine dehydrogenase (EC 1.5.1.16) activities in the tumors and roots were determined by a microassay developed by Otten and Schilperoort (30).

RESULTS

Localization of *Kpn*I and *Xba*I restriction enzyme recognition sites. To facilitate more precise physical mapping of the Ti plasmid insertions, the locations of the recognition sites of the restriction enzymes *Kpn*I and *Xba*I were determined relative to a published *Sma*I-*Hpa*I map (7). Double digestions with the restriction endonucleases *Sma*I, *Hpa*I, *Kpn*I, and *Xba*I were performed on plasmid DNA of Ti plasmid deletion mutants (29). The gel electrophoretic patterns of Ti plasmid DNA treated with each of the enzymes are shown in Fig. 1. The fragments are numbered according to their relative mobilities during electrophoresis. The molecular weights of the double-digested fragments and fusion fragments of the DNA from deletion mutants were determined by their mobilities relative to those of *Sma*I Ti plasmid fragments of known molecular weight (7). Since DNA fragments of a high molecular weight ($>10 \times 10^6$) show nearly the same mobility in 0.7% agarose gels (15), the molecular weights of large fragments generated by *Kpn*I and *Xba*I were deduced from the distances between restriction enzyme recognition sites on the map. The numbers and estimated molecular weights of the fragments generated by each of the four enzymes are given in Table 1. Fragments with molecular weights lower than 0.7×10^6 would normally not have been detected. By adding the molecular weights of the restriction endonuclease fragments, a total molecular weight for the Ti plasmid of 121.3×10^6 was calculated as determined by Chilton et al. (7).

The Ti plasmid from *A. tumefaciens* strain Ach5 used in this study differed slightly from the Ti plasmid harbored by strain B6-806 in that under our conditions the doublets *Sma*I 14ab and 16ab could be resolved in two separated bands. In addition, on the basis of deletion analysis, the map positions of *Sma*I fragments 12 and 14a were interchanged (29), correcting the published data for B6-806 (7).

By conventional methods we could construct

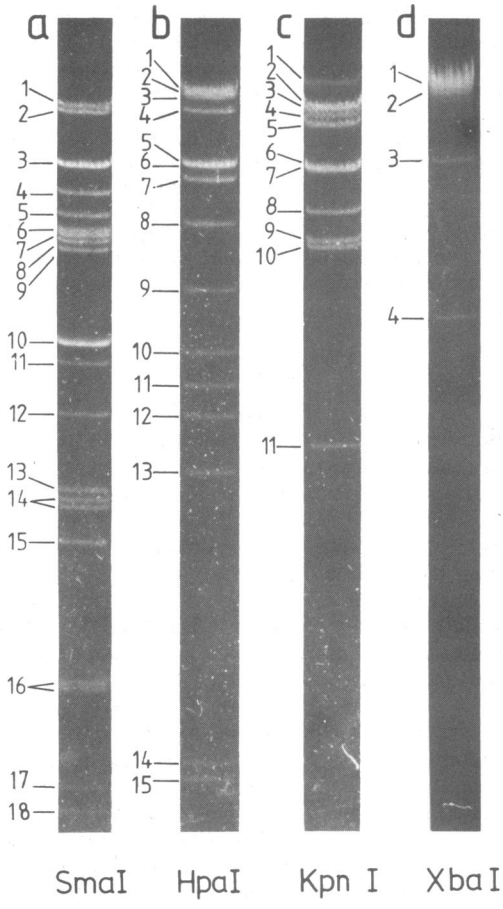

FIG. 1. *Restriction endonuclease fingerprints of Ach5 Ti plasmid DNA and the band numbers of the generated fragments after electrophoresis in 0.7% agarose gels. LBA4001 Ti plasmid DNA was treated with SmaI (a), HpaI (b), KpnI (c), and XbaI (d). SmaI fragments 3 and 10 are a doublet and a triplet, respectively, and XbaI fragments 1 and 4 are both doublets. (see also Table 1).*

a *Kpn*I map and an *Xba*I map relative to the *Sma*I-*Hpa*I map for Ti plasmid deletion mutants isolated previously (29) and for the whole Ti plasmid. First, a map was constructed for the simplest Ti plasmid deletion mutants (having the largest deletions), and with the use of successively larger Ti plasmid deletion mutants (smaller deletions), a *Kpn*I-*Xba*I map could be constructed for these mutants (data not shown) and finally for the whole Ti plasmid. The locations of the endonuclease fragments could be confirmed by some of the Tn*904* insertions described in this paper and by the analysis of Tn*1831* Ti plasmid insertion mutants and newly isolated Ti plasmid deletion mutants (manuscripts in preparation).

From a partial digestion of pTi Ach5 DNA with *Xba*I, we deduced that an *Xba*I fragment (fragment 5) of 0.5 megadaltons must be present between a 4.3-megadalton fragment and a 9.9-megadalton fragment, as faint bands of 4.8 and about 10.4 megadaltons appeared (Fig. 1). This fragment was placed at the indicated position because in this way the molecular weights of double-digested fragments could best be explained. The map positions of the recognition sites for the endonucleases *Hpa*I, *Sma*I, *Kpn*I, and *Xba*I on pTi Ach5 are shown in Fig. 2.

Selection of Tn*904* insertion mutants. Klapwijk et al. (27) isolated 155 stable Ti plasmid Tn*904* insertion mutants by using a transfer-deficient derivative of RP1-pMG1 to donate Tn*904* to a constitutive self-transmissible Ti plasmid via conjugative transfer and selection on streptomycin. The presence of a Tn*904*-mutated Ti plasmid was confirmed by the introduction via conjugation of an incompatible Ti plasmid, which resulted in the loss of the original resident Tn*904* mutated Ti plasmid.

For this study strains were selected from the Tn*904* Ti plasmid insertion mutants with altered tumor-inducing properties and mutants without alterations of known Ti plasmid-coded characteristics. The selected strains originated usually from different independent conjugation experiments, and selection of more than one strain per conjugation experiment was only possible when clear phenotypic differences existed between the strains. It is noted that in the previous study (27) no mutants were isolated affected in other known Ti plasmid-coded functions, such as octopine catabolism, synthesis of octopine in induced tumors, and Ti plasmid transfer functions. All analyzed mutant strains were tested for their abilities to induce crown gall tumors on petunia, tomato, and *K. daigremontiana*. These plants were chosen because they appeared to react differently to some *A. tumefaciens* strains tested. Two T-DNA deletion mutants, for instance, which were described previously (29) as being weakly virulent on kalanchoe, later on appeared to be avirulent on tomato, but were found to induce tumors on petunia, although the tumors were small.

We were unable to isolate Ti plasmid DNA from all selected mutant strains; as a consequence of this, two virulence-affected strains and some strains that were normally virulent could therefore not be analyzed with the followed procedure. The phenotypes of tumors induced on *K. daigremontiana* stems by strains LBA4205(pAL223), LBA4210(pAL228), LBA4228(pAL245), and LBA4219(pAL237) are shown in Fig. 3.

The tumors induced by LBA4205(pAL223) were of normal morphology. LBA4210(pAL228)

TABLE 1. *Band positions and estimated molecular weights of restriction fragments*[a]

Restriction enzyme	No.	Mol wt[b]	Restriction enzyme	No.	Mol wt[b]
*Sma*I	1	14.9		8	6.54
	2	14.0		9	4.8
	3ab	9.2		10	3.95
	4	7.5		11	3.6
	5	6.6		12	3.3
	6	6.05		13	2.85
	7	6.0		14	1.38
	8	5.7		15	1.33
	9	5.5		16	0.88
	10abc	4.0			
	11	3.78	*Kpn*I	1	24.6
	12	3.22		2	15.2
	13	2.65		3	14.8
	14	2.5		4	14.0
	15	2.28		5	13.0
	16	1.58		6	9.2
	17	1.23		7	9.1
	18	1.17		8	6.8
	19	0.88		9	6.0
	20	0.67		10	5.7
	21	0.64		11	2.9
*Hpa*I	1	18.9	*Xba*I	1a	40.0
	2	16.4		1b	38.4
	3	15.5		2	24.0
	4	14.4		3	9.8
	5	9.4		4ab	4.3
	6	9.4		5	0.5
	7	8.7			

[a] The molecular weights of *Sma*I and *Hpa*I fragments are according to Chilton et al. (7). Doublets and triplets in *Sma*I and *Xba*I digests are indicated by a, b, and c following the fragment number (see also Fig. 1).
[b] Estimated molecular weight (10^6) of the restriction fragments.

induced small tumors on *K. daigremontiana* with more adventitious root formation than normal. LBA4228(pAL245) induced very small tumors on tomato and kalanchoe. Strain LBA4219(pAL237) is representative of mutants that did not induce tumors on the plant species used. The tumor-inducing properties of all strains studied are summarized in Table 2. It can be seen that compared with the wild type, several mutants showed differences in their tumor-inducing capacities on different plant species. Strains LBA4210(pAL228) and LBA4228(pAL245) induced small tumors on petunia but also formed small tumors on both tomato and kalanchoe. Strain LBA4212(pAL230) was selected because of its weaker tumorigenic capacity specifically on tomato. The tumors formed were, however, not as small as those induced by LBA4228(pAL245). All described virulent insertion mutants induced tumors that contained D-lysopine dehydrogenase activity.

Tumor phenotype and Tn*904* insertion position. The insertion of a translocatable element involves the linear insertion of an intact, discrete, nonpermuted sequence of DNA. The ends of a transposon are involved in the transpositional process (19, 28). The results of the restriction analysis concerning the Tn*904* insertion sites in pTi Ach5 are summarized in Table 2. The insertion positions were determined by comparing the gel electrophoretic patterns of the wild-type Ti plasmid and mutated Ti plasmids after treatment with restriction endonucleases. Plasmid DNA from each mutant strain contained an insertion of additional DNA. This provides support for the genetic evidence that the transposon is located on the Ti plasmid (27). Ti plasmid pAL245 contained two insertions of Tn*904* in different places. This could be deduced from the fact that two Ti plasmid fragments present in a *Sma*I digest of the wild-type Ti plasmid were missing, whereas four new fragments were generated by *Sma*I. A comparable result was obtained for mutant LBA4212(pAL230). The avirulent mutants LBA4219(pAL237), LBA4220(pAL238), LBA4226(pAL243), and LBA4232(pAL248) carried insertions in *Hpa*I fragment 12 of the Ti plasmid. This result shows that this region of the Ti plasmid harbors genes which are essential for

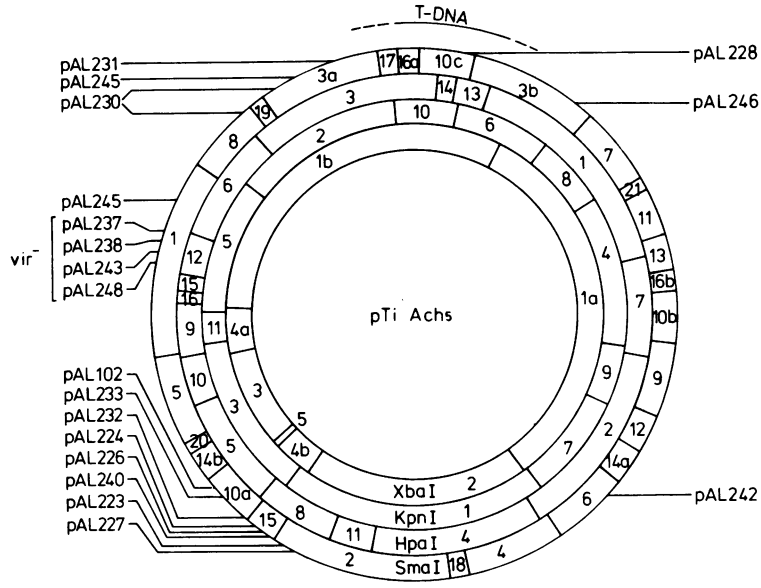

FIG. 2. *Restriction endonuclease map of the Ach5 Ti plasmid insertion positions of transposon Tn904. A KpnI map and an XbaI map are shown relative to a SmaI-HpaI map. Each plasmid designation refers to a specific mutated Ti plasmid due to a Tn904 insertion at the indicated position. A region essential for tumorigenicity is indicated as vir⁻, and the T-DNA region for tobacco tumors induced by strain Ach5 is shown (unpublished data). The T-DNA region is comparable to the T-DNA region detected in tobacco tumors induced by strain A6 as published by Thomashow et al. (41).*

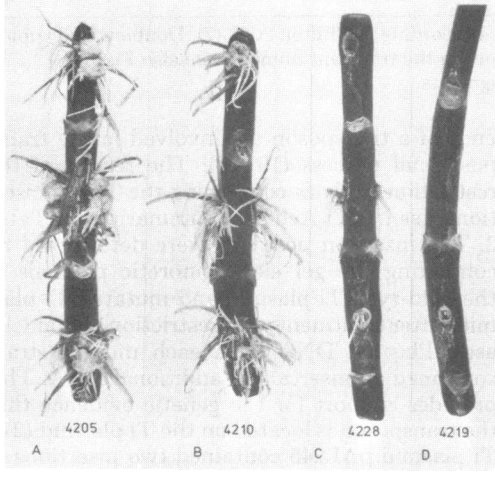

FIG. 3. *Tumors on K. daigremontiana stems. Wounds on stems of K. daigremontiana plants were infected with suspensions of Ti plasmid mutants of A. tumefaciens: LBA4205(pAL223) (A), LBA4210-(pAL228) (B), LBA4228(pAL245) (C), and LBA4219-(pAL237) (D). One strain was tested per plant.*

tumor formation. The weakly virulent mutant strain LBA4228(pAL245) had one of the Tn*904* insertions in *Hpa*I fragment 6, close to *Hpa*I fragment 12 (Fig. 2). The other Tn*904* transposon in pAL245 was located in *Sma*I fragment 3a.

It is difficult to decide which of the mutations caused weak virulence. LBA4212(pAL230) was less virulent than the wild type. In this case Tn*904* was inserted in *Sma*I fragment 8 and in *Sma*I fragment 3a; also in this case it is difficult to decide which of the mutations caused weak virulence. It can be seen from Table 2 that strain LBA4210(pAL228) had an insertion in restriction enzyme fragment *Hpa*I-13, which is part of the common sequence present in all types of Ti plasmids in the T-DNA region. Most likely this mutation caused the reduced size of the tumors on *K. daigremontiana* and the excessive root formation. Mutant strain LBA4229(pAL246) was of interest because the insertion of Tn*904* on its plasmid was in *Sma*I fragment 3b, which is close to or possibly in the T-DNA region. The latter possibility, however, turned out not to be true, because analysis of sterile callus tissue derived from an excised tumor induced by LBA4229(pAL246) on tobacco showed that only T-DNA to the left of the *Hpa*I endonuclease site between fragments 13 and 1 was present (unpublished data).

Eleven independently isolated and randomly chosen mutant strains, which were not affected in virulence or other known phenotypic traits (23), were analyzed for map position in order to know more about the insertion behavior of this transposon. It can be seen in Fig. 2 that 8 of the

TABLE 2. *Virulence of A. tumefaciens mutant strains and characteristics of their mutated Ti plasmids*[a]

Strain	Virulence[b]			Plasmid	Fragment of modified mobility			Mol wt (10^6) of:	
	Petunia	Tomato	Kalanchoe		*Sma*I	*Hpa*I	*Kpn*I	New fragments[c]	Additional DNA[d]
LBA4203	+	+	+	pAL102	10a	5		6.0, 4.9	6.9
LBA4205	+	+	+	pAL223	2	8		14.9, 5.8	6.7
LBA4206	+	+	+	pAL224	15	8		9.2, 4.0	10.9
LBA4208	+	+	+	pAL226	15			3.8, 8.7, 4.2	14.4
LBA4209	+	+	+	pAL227	2	8		15.9, 4.9	6.8
LBA4210	±	±	±[e]	pAL228	10c	13	10	6.2, 4.8	7.0
LBA4212	+	+[f]	+	pAL230	3a	3	2	10.8, 5.5	7.1
				pAL230	8	3	2	8.0, 5.0	7.3
LBA4213	+	+	+	pAL231	3a	3	2	12.5, 4.8	8.1
LBA4214	+	+	+	pAL232	10a	8	1	7.0, 4.0	7.0
LBA4215	+	+	+	pAL233	10a	5		7.6, 6.4, 4.1	14.1
LBA4219	−	−	−	pAL237	1	12	5	12.2, 7.7, 4.3	9.3
LBA4220	−	−	−	pAL238	1	12		12.5, 8.4, 8.6	14.6
LBA4223	+	+	+	pAL240	15	8		5.3, 4.1	7.1
LBA4225	+	+	+	pAL242	6	2		7.3, 4.1	5.4
LBA4226	−	−	−	pAL243	1	12		11.5, 11.0	7.6
LBA4228	±	±	±	pAL245	1	6	5	16–18, 4.5	ca. 7
				pAL245	3a	3	2	9.7, 6.6	7.1
LBA4229	+	+[e]	+	pAL246	3b	1	6	10.5, 5.5	6.8
LBA4232	−	−	−	pAL248	1	12		12.0, 10.0	7.1

[a] Separate strain and plasmid designations are given to mutant strains to distinguish between the bacterium as a biological entity and a plasmid as a physical structure not necessarily connected with a certain host strain.

[b] +, Virulent; ±, weakly virulent; −, aviruent.

[c] Molecular weights of the new restriction fragments were estimated from gel electrophoresis patterns of *Sma*I-digested mutant Ti plasmid DNA.

[d] Additional DNA was calculated as the difference between the molecular weight of new generated *Sma*I fragments and the mutated *Sma*I fragment in which the insertion took place (see also Fig. 5).

[e] Excessive root formation was observed (see Fig. 3).

[f] Possibly slightly altered infectivity (see text).

11 mutants contained plasmids with insertions in a region formed by *Hpa*I fragment 8 and/or *Sma*I fragment 10a. The Tn*904* insertion in the other three mutants [LBA4225(pAL242), LBA4213(pAL231), and LBA4229(pAL246) (see above)] were localized in *Sma*I fragments 3a, 6, and 3b, respectively. These data suggest that Tn*904* preferentially inserts in a region close to the origin of replication (29), but that insertions at other positions also occur.

The variable size of Tn*904* insertions. Not much about the structure and the biological functions of Tn*904* in incompatibility P1 plasmid RP1-pMG1 has been published. It has been reported that this R-plasmid has a molecular weight that is at least 6.0×10^6 more than that of RP1 (25). Restriction enzyme analysis of Tn*904*-mutated Ti plasmids yielded additional data about Tn*904* transpositions. In Table 2 the estimated molecular weights are given of restriction fragments that are present in the *Sma*I fragmentation pattern of Ti plasmids with Tn*904* insertions which are absent from the wild-type Ti plasmid restriction fragmentation pattern. Gel electrophoretic patterns for a number of such mutants are shown in Fig. 4. Figure 5 summarizes the frequencies with which additional amounts of DNA with certain molecular weights occurred in the mutants. It can be seen that in most cases the molecular weight of the extra DNA was approximately 7×10^6. From the data in Table 2, it can be concluded that with this extra DNA, one recognition site for restriction enzyme *Sma*I was introduced. It is apparent that sometimes a double amount of DNA, ca. 14×10^6, was inserted and that at the same time a second *Sma*I site was introduced. This suggests that in these cases Tn*904* was integrated as two linked copies. Values between 8×10^6 and 14×10^6 for the inserted DNA were found for LBA4206(pAL224), LBA4213(pAL231), and LBA4219(pAL237). LBA4206(pAL224) and LBA4213(pAL231) did obtain the extra *Sma*I restriction site, whereas LBA4219(pAL237) obtained two extra sites. The summary in Fig. 5 clearly demonstrates that Tn*904* was not always

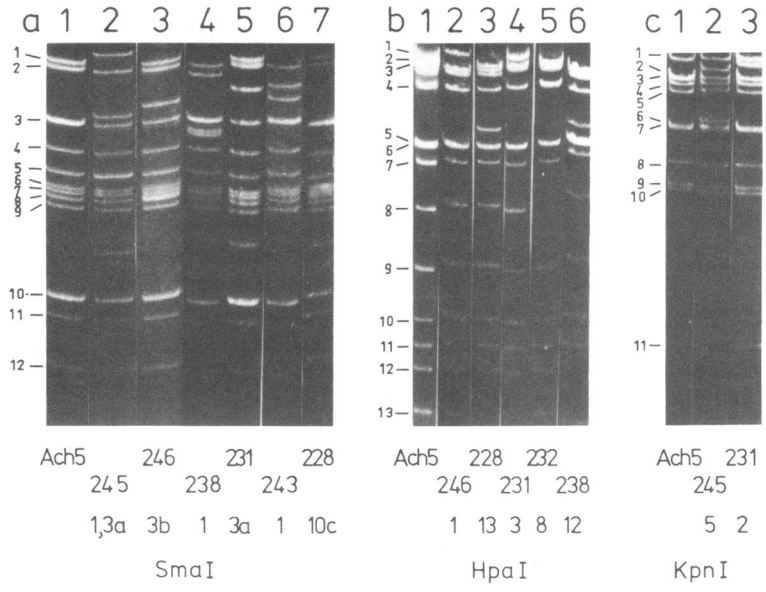

FIG. 4. *Restriction endonuclease fingerprints of Ti plasmids. Plasmids with different Tn904 insertions were treated with the restriction enzymes SmaI (a), HpaI (b), and KpnI (c). The plasmids treated with SmaI were pTi Ach5 (1), pAL245 (2), pAL246 (3), pAL238 (4), pAL231 (5), pAL243 (6), and pAL228 (7); those treated with Hpa I were pTi Ach5 (1), pAL246 (2), pAL228 (3), pAL231 (4), pAL232 (5), and pAL238 (6); and those treated with KpnI were pTi Ach5 (1), pAL245 (2), and pAL231 (3). The properties of strains harboring these plasmids are summarized in Table 2.*

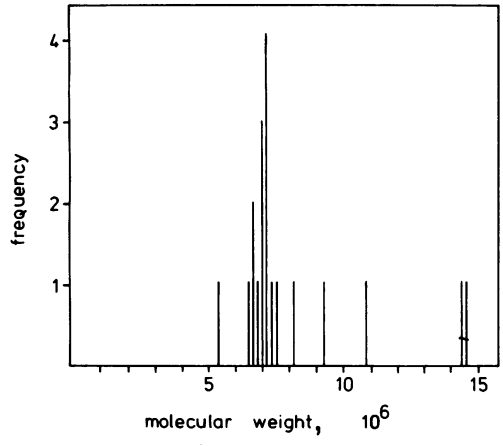

FIG. 5. *Additional molecular weight associated with insertions of Tn904. The data are summarized in Table 2.*

present as a discrete entity in the Ti plasmid. Insertions that caused the loss of an endonuclease recognition site were not observed; e.g., the Tn*904* insertion of LBA4210(pAL228) in *Hpa*I fragment 13 appeared not to affect *Hpa*I fragment 14. Therefore, no large deletions in the Ti plasmid were generally generated concomitant with the insertion of Tn*904*.

DISCUSSION

The position and nature of Tn*904* insertions. A *Kpn*I-*Xba*I restriction endonuclease map was constructed relative to a published *Sma*I-*Hpa*I map (7) in order to analyze more accurately and unambiguously Ti plasmid insertion and deletion mutants. We screened commonly used restriction endonucleases in order to find enzymes recognizing relatively few sites in the Ti plasmid that could be mapped using relatively simple conventional techniques in combination with previously described deletion mutants (29). It could be expected that such enzymes would in addition be helpful for T-DNA analysis in tumor cells. Both *Kpn*I and *Xba*I were found to give relative simple patterns, and although *Xba*I was not used to determine Tn*904* insertion positions, the *Xba*I map has proved already to be helpful in analyzing Ti plasmid deletion mutants (manuscript in preparation), and it is, to our knowledge, the only restriction endonuclease that does not have a recognition site in Ti plasmid DNA detected in crown gall tumors (T-DNA) as described by Thomashow et al. (41) for octopine tumor line A6.

It should be noticed that minor variations in distances between the relative recognition sites for all four enzymes, as given in Fig. 2, might exist. For a number of sites the method used

does not allow an accuracy of more than 0.5 × 10^6. *Kpn*I fragments 2 and 3 were not well resolved, and they are numbered according to their molecular weights measured from the map. In neither of the previous reports (7, 29) were the precise locations of *Hpa*I fragments 12, 15, and 16 determined relative to *Sma*I fragment 1. We were not able to improve this either by *Sma*I restriction patterns of Ti plasmids with Tn*904* inserted in *Hpa*I fragment 12 or by double digestions with *Kpn*I and *Xba*I.

From the Ti plasmid Tn*904* insertion mutants evidence was obtained that Tn*904* did not insert as a discrete translocatable element and that in two strains, LBA4228(pAL245) and LBA4212(pAL230), the Ti plasmid contained two insertions of Tn*904* at different positions. A nondiscrete translocation of DNA was reported previously for R-determinants of certain R-plasmids, R100 in *Proteus mirabilis* (33) and DS-5C3 in *Streptococcus faecalis* (8), and for Tn*1771* (37) and Tn*1721* (32). If the nondiscrete size of Tn*904* were the result of a double insertion of Tn*904* followed by a deletion within the transposon, it could be expected that restriction analysis with *Sma*I should give a fragment of constant size for insertions with high molecular weights, because *Sma*I has two recognition sites in a 14 × 10^6 Tn*904* insert and one site in a 7 × 10^6 insert. This was not observed, however. An alternative explanation for the variable insertion size of Tn*904* could be that the transposon contains a streptomycin resistance gene that is capable of amplification by forming tandem repeats, as has been found for Tn*1721* (32) and Tn*1771* (37).

It has been reported that deletions generated by Tn*A* retained the Tn*A* region intact, and the formation of the deletions was *recA* dependent (22). Although no Ti plasmid restriction sites were missing, it cannot be excluded that small regions of the Ti plasmid in which no restriction sites are located were deleted, as the mutants were isolated in a Rec^+ host. At this moment we can only speculate as to the most probable mechanism by which the size variation of Tn*904* arose. Figure 2 shows that insertion of Tn*904* into the Ti plasmid occurs preferentially in a region close to the replicator, as defined by Koekman et al. (29; B. P. Koekman, P. J. J. Hooykaas, and R. A. Schilperoort, Plasmid, in press). However, Tn*904* insertions in regions of the Ti plasmid involved in conjugational transfer have been excluded as a consequence of the isolation procedure. The distribution of Tn*904* insertions helps us to understand why no mutants affected in octopine catabolism functions were isolated. It is known that both octopine catabolism functions and transfer functions are located at the right side of the Ti plasmid (29). Moreover, these functions share a common negative control mechanism. A high percentage of mutants affected in octopine catabolism are also transfer deficient (26). In addition to the suggested region of preferential integration and/or a possible common negative influence on both transfer and octopine catabolism functions, it is noted that the number of insertion mutants isolated (155) is relatively low in relation to the size of the Ti plasmid (121. 3 Mdal).

The results, however, indicate that it is probably more useful to study Ti plasmid functions with other techniques or other mutagens that might give complementary information rather than to isolate more Tn*904* Ti plasmid mutants. Mutant strains LBA4228(pAL245) and LBA-4212(pAL230) are remarkable in that they carry two Tn*904* insertions. Although it has been suggested that plasmids containing a transposon are immune to the insertion of its second copy (32), it has been shown that one plasmid is able to carry two transposons (2, 12, 31) that are inserted in opposite orientations. It is unclear whether the two transposons in LBA4228-(pAL245) and LBA4212(pAL230) inserted simultaneously or whether one insertion occurred under the high selective pressure used (1,500 mg of streptomycin per liter) and generated a copy at another position. A comparable result was obtained with Tn*A* transposed under high selective pressure into the R6K plasmid (22).

Characteristics of Tn*904* insertion mutants. Four mutants were described with Tn*904* insertions in *Hpa*I fragment 12, and therefore this region of the Ti plasmid is involved in tumor formation.

The function of the mutated region is not clear, but one of the possibilities is that it carries genes of which the expression in the bacterium would be essential for the process of tumor formation, because the mutated DNA is located far away from the T-DNA region and no DNA from the mutated region has ever been detected in plant cells. Such a bacterial function could be involved in the transfer of Ti plasmid genes into the plant cells. It is important to emphasize that these avirulent mutants were not affected in functions for bacterial conjugation. Another possible function of the mutated Ti plasmid region could be a role in the processing of Ti plasmid genes in the plant cell.

The weak virulence of strain LBA4228-(pAL245), with a Tn*904* insertion in *Hpa*I fragments 6 and 3, may be due to a lower frequency of tumor cell initiation. It is known that tumor size is dependent on the number of bacteria in wounds of identical size, i.e., when the same number of plant cells are exposed to the bac-

teria. The size of the tumors induced by LBA4228(pAL245) when applied directly after wounding is the same as that of tumors scored at the same time but induced by the wild-type strain either with a lower number of bacteria per milliliter (10^4 to 10^5) or with an equal concentration of wild-type bacteria, but 3 to 4 days after wounding the plant, i.e., after the optimal sensitive period for tumor induction, when fewer susceptible cells are present (3). The same reasoning can be followed for mutant strain LBA4212(pAL230), which shows less virulence on tomato than does the wild-type strain, but normal virulence on kalanchoe and petunia. The results obtained with LBA4228(pAL245) and LBA4212(pAL230) can both be explained by a polar effect of Tn*904* on the expression of the virulence region in *Hpa*I fragment 12, although it is noted that both mutants harbor two Tn*904* Ti plasmid insertions.

In agreement with the observation on the avirulent mutants are the results of homology studies, showing the presence of a conserved region of DNA in Ti plasmids of different types. *Hpa*I fragment 12 is part of *Sma*I fragment 1, which has a high degree of homology with the DNAs of several nopaline Ti plasmids and a null-type Ti plasmid (13, 20).

Analogously, studies with the nopaline Ti plasmid pTi C58 showed that mutations in DNA with a high degree of homology between several types of Ti plasmids do affect tumor formation (23). A direct comparison between analogous regions of homology between pTi Ach5 and pTi C58—outside the T-DNA—is impossible because of lack of data about the mutual location of the homologous regions. Insertion mutant LBA4210(pAL228) has an insertion in *Hpa*I fragment 13, which is part of the T-DNA region and induces tumors with excessive root formation on *K. daigremontiana*. This mutant clearly shows that a mutation in the common sequence of the octopine Ti plasmid does not invariably lead to avirulence, as has been reported (11, 34). The nature of this tumor phenotype has been investigated in greater detail in combination with other T-DNA region insertion mutants that give rise to tumors on kalanchoe without root formation at all. A combination of the phenotypes of tumors induced on several plant species by these two types of morphology mutants, mixed infections, the effect of the addition of plant hormones during infection, and phenotypes of growing tobacco tissue under sterile conditions in tissue culture obtained from excised tumors indicates an active role for T-DNA in the control of phytohormone levels in crown gall cells (manuscript in preparation). This idea is supported by the detection of the inserts in crown gall cells (manuscript in preparation) and by tissue culture experiments from which it is known that the development of roots or shoots from normal plant tissue in culture is regulated by the relative concentrations of auxins and cytokinins in the culture medium (39). Besides that, it is known that tumor cells grow under tissue culture conditions without phytohormones on the culture medium, whereas normal cells, growing as amorphous tissue, need balanced amounts of phytohormones for vigorous growth (1, 4).

The morphology of tumors induced by LBA4210(pAL228) (excessive root formation) can therefore, in our opinion, best be explained by relatively lower concentrations of active cytokinin-like activities to concentrations of physiologically active auxin-like activities in the tumor in comparison with a wild-type tumor.

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