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The Hypervirulence of *Agrobacterium tumefaciens* A281 Is Encoded in a Region of pTiBo542 Outside of T-DNA

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We used a binary-vector strategy to study the hypervirulence of *Agrobacterium tumefaciens* A281, an L,L-succinamopine strain. Strain A281 is hypervirulent on several solanaceous plants. We constructed plasmids (pCS65 and pCS277) carrying either the transferred DNA (T-DNA) or the remainder of the tumor-inducing (Ti) plasmid (pEHA101) from this strain and tested each of these constructs in *trans* with complementary regions from heterologous Ti plasmids. Hypervirulence on tobacco could be reconstructed in a bipartite strain with the L,L-succinamopine T-DNA and the *vir* region on separate plasmids. pEHA101 was able to complement octopine T-DNA to hypervirulence on tobacco and tomato plants. Nopaline T-DNA was complemented better on tomato plants by pEHA101 than it was by its own nopaline *vir* region, but not to hypervirulence. L,L-Succinamopine T-DNA could not be complemented to hypervirulence on tobacco and tomato plants with either heterologous *vir* region. From these results we suggest that the hypervirulence of strain A281 is due to non-T-DNA sequences on the Ti plasmid.

Agrobacterium tumefaciens is the etiologic agent of crown gall. Oncogenic strains of *A. tumefaciens* harbor large plasmids called Ti (tumor-inducing) plasmids (54–56). Part of the Ti plasmid—the transferred DNA (T-DNA)—is transferred to the plant, where it is stably maintained in the nuclear DNA (9, 10). The T-DNA contains genes that are transcribed in the plant (8), encoding enzymes for opine synthesis (5, 21, 38, 45, 51) and phytohormone synthesis (1, 3, 50, 53). A second region of approximately 30 to 40 kilobase pairs (kbp) of the Ti plasmid that is involved in tumorigenesis but is not maintained in the tumor is called the virulence (*vir*) region. Drummond and Chilton (17) found extensive regions of DNA that were conserved on several wide-host-range Ti plasmids including octopine, nopaline, and L,L-succinamopine types. In an elegant study, Engler et al. (18) precisely defined these areas of homology between the *vir* regions of octopine and nopaline Ti plasmids. The *vir* regions from wide-host-range octopine (26, 36, 39, 40, 48) and nopaline (32, 43) Ti plasmids, as well as from a narrow-host-range octopine Ti plasmid (41), have been studied by mutagenesis and genetic complementation. The pTiC58 (nopaline) *vir* region contains six genetically defined complementation groups, one of which is *cis* dominant (43). Klee et al. (40) defined five complementation groups (*virA* through *virE*) for an octopine plasmid. The latter were extended to include *virO* and *virF* (27, 34). Many mutations in either of these Ti plasmids can be complemented by a heterologous wild-type Ti plasmid or mutations in other loci from heterologous Ti plasmids (34). Some mutations in the octopine *vir* region can be complemented by the root-inducing plasmid of *A. rhizogenes*, indicating conservation of *vir* region functions (34). A few gene products from the *virC* region of pTiC58 have been identified (23).

Hoekema et al. (28) and de Framond et al. (14) demonstrated that the Ti plasmid could be divided into two separately replicating plasmids in the same bacterium and still induce tumors similar to those induced by the wild type on the host plants tested. Hoekema et al. (29) then tested virulence genes for cross-functionality in a study using this binary system. Octopine T-DNA could be transferred to a variety of plants by the virulence regions of a root-inducing plasmid, a limited-host-range plasmid, and a wild-type nopaline plasmid, but not by *Rhizobium* sp. symbiotic plasmids. Otten et al. (48) showed by transient octopine assays that mutations in the *virE* locus of pTiB6S3 can be complemented by coinfection with wild-type *Agrobacterium* strains, consistent with the view that the product(s) of the *virE* locus must be diffusible.

We report here a study of the hypervirulence of *A. tumefaciens* A281 on solanaceous plants. Our experiments addressed the question of whether this phenotype is a property of the T-DNA or non-T-DNA portion of the Ti plasmid. Our strategy was to divide the pTiBo542 plasmid (from A281) into a binary system and assay the T-DNA and *vir* regions with complementary regions from heterologous Ti plasmids. We found that interactions of T-DNAs and *vir* regions were complex and exhibited different responses on different host plants. However, we could conclude that the hypervirulence of strain A281 on solanaceous plants is a property of the non-T-DNA portion of the Ti plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in these experiments are listed in Tables 1 and 2, together with the characteristic tumor opines for *A. tumefaciens* strains. The chromosomal background for all *A. tumefaciens* strains is C58 (a Ti plasmid-cured nopaline strain) except for Bo542, which has a Bo542 chromosomal background, and Ach5 and all LBA strains, which have an Ach5 chromosomal background. Strain ASE-1 is a T-DNA deletion derivative of pTiT37, the nopaline Ti plasmid in strain A208. A description of its construction will be published elsewhere (S. G. Rogers et al., manuscript in preparation). The probes used on the blots (see Fig. 2), pEHC277, pEHC293, and pEHC180, are cosmid clones of portions of pTiBo542 (33). pEHC277 contains *Bam*HI fragments from the T-DNA region, and pEHC180 and pEHC293 contain fragments to the left of the T-DNA

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TABLE 1. *A. tumefaciens* strains and plasmids used in this work and their relevant characteristics

Strain	Relevant plasmid(s)	Plasmid characteristic		Source or reference
		Marker[a]	Tumor opine[b]	
Whole Ti plasmid strains				
A281	pTiBo542	None	L,L-SAP, LOP, AGR	52
A208	pTiT37	None	NOP, AGC	52
Ach5	pTiAch5	None	OCT, AGR	24
Bo542 Tempé	pTiBo542	None	L,L-SAP, LOP	52
Control strains				
A479	None	None	Not applicable	E. W. Nester
ASE-1	pASE-1	Km	None	S. G. Rogers
LBA4404	pAL4404	None	None	47
EHA101	pEHA101	Km	None	This work
A479(pCS277)	pCS277	Cb	None	This work
A479(pCS65)	pCS65	Cb	None	This work
A479(pAL4404::Tn*903*)	pAL4404::Tn*903*	Km	None	A. de Framond
Bipartite strains				
Nopaline *vir* region				
ASE-1(mini Ti/pRK)	pASE-1	Km		This work
	Mini Ti/pRK	Cb, Km	NOP, AGC	
ASE-1(pCS65)	pASE-1	Km		This work
	pCS65	Cb	L,L-SAP, LOP, AGR	
Octopine *vir* region				
LBA4434	pAL4404	None		28
	pAL1050	Cb, Km	OCT, AGR	
LBA4404(mini Ti/pRK)	pAL4404	None		14
	Mini Ti/pRK	Cb, Km	NOP, AGC	
LBA4404(pCS65)	pAL4404	None		This work
	pCS65	Cb	L,L-SAP, LOP	
A479(pAL4404::Tn*903*)(pCS277)	pAL4404::Tn*903*	Km		This work
	pCS277	Cb	L,L-SAP, LOP, AGR	
A479(pAL4404::Tn*903*)(pCS65)	pAL4404::Tn*903*	Km		This work
	pCS65	Cb	L,L-SAP, LOP	
L,L-Succinamopine *vir* region				
EHA101(pCS277)	pEHA101	Km		This work
	pCS277	Cb	L,L-SAP, LOP, AGR	
EHA101(pCS65)	pEHA101	Km		This work
	pCS65	Cb	L,L-SAP, LOP	
EHA101(mini Ti/pRK)	pEHA101	Km		This work
	Mini Ti/pRK	Cb, Km	NOP, AGC	
EHA101(pAL1050)	pEHA101	Km		This work
	pAL1050	Cb, Km	OCT, AGR	

[a] Km, Kanamycin; Cb, carbenicillin; Sp, spectinomycin; Sm, streptomycin; Tc, tetracycline; Gm, gentamicin.
[b] L,L-SAP, L,L-Succinamopine; LOP, leucinopine; AGR, agropine; NOP, nopaline; AGC, agrocinopine; OCT, octopine.

region. pRK325 is a shuttle vector plasmid derived from pRK290 (G. L. Helmer et al., manuscript in preparation). A 700-base-pair piece from pBR325 was cloned into the unique *Eco*RI site of pRK290 to allow cointegration of pBR-derived cloning vectors containing inserts of interest. This allowed replication of these pBR-based clones in *A. tumefaciens*.

Routine growth conditions for *A. tumefaciens* and *Escherichia coli* were as described previously (33). In some instances, *A. tumefaciens* was grown on AT minimal medium (22). Antibiotic concentrations were as follows. For *A. tumefaciens*, we used kanamycin sulfate (Sigma Chemical Co., St. Louis, Mo.), 50 μg/ml; naladixic acid (Sigma), 25 μg/ml; tetracycline hydrochloride (Calbiochem-Behring, La Jolla, Calif.), 10 μg/ml; and carbenicillin (or ampicillin) (Sigma), 50 μg/ml; and spectinomycin, 25 μg/ml. For *E. coli*, we used kanamycin sulfate, 50 μg/ml; tetracycline hydrochloride (Sigma), 25 μg/ml; carbenicillin (or ampicillin) (Sigma), 25 μg/ml; and spectinomycin, 25 μg/ml.

Triparental matings. Matings among *E. coli* or *Agrobacterium* strains were achieved by mixing 0.1 ml each of overnight cultures of a donor strain (always an *E. coli* strain), a recipient strain (either an *E. coli* or an *Agrobacterium* strain), and a helper strain—*E. coli* HB101 (pRK2013). All overnight cultures were grown in the appropriate antibiotics, centrifuged, and resuspended in L broth before being mixed for matings. Mating mixture, (100 μl) was spread on L agar plates without antibiotics for 5 to 10 (*E. coli*) or 24 to 48 (*A. tumefaciens*) h. *E. coli* transconjugant strains were selected on L agar, and *Agrobacterium* transconjugant strains were selected on AT minimal medium, each containing the appropriate antibiotics. The *E. coli* strains used in these experiments did not grow on AT minimal medium. Selected colonies were purified by three successive single-colony isolations on selection medium. After purification, all strains were maintained on L agar containing the appropriate antibiotics. Strains were verified by viewing mini-prep DNA on agarose gels (see below). *Agrobacterium* strains were further verified by screening for

TABLE 2. *E. coli* strains and plasmids used in this work and their relevant characteristics

Strain or plasmid	Relevant plasmid or plasmid marker(s)[a]	Relevant characteristic(s)	Source or reference
LE392	None		46
HB101	None		7
SR43(mini Ti/pRK)	Mini Ti/pRK		A. de Framond
JM101(pAL1050)	pAL1050		A. Hoekema
pRK2013	Km	Mobilizing plasmid	15
pRK325	Tc	Shuttle vector pRK290 + pBR homology	G. Helmer, in preparation
pMO25	Tc	pRK252 + M13 *ori* and packaging site	W. Barnes
pMO25-E°	Tc	pMO25 minus *Eco*RI site	This work
R751-pMG2	Gm	P1 eviction plasmid	37
pMON249	Tc, Cb, Km[b]	Km^r gene source for pEHK2	S. G. Rogers
pEHC277	Cb	*Bam*HI fragments 5, 10, 13b, 26, 33, 22, 16, 18, and 7b	33
pEHC293	Cb	*Bam*HI fragments 21a, 13c, 9, 21b, 15, and 1	33
pEHC180	Cb	*Bam*HI fragments 21b, 15, 1, and 5	33

[a] Abbreviations: Km, kanamycin; Cb, carbenicillin; Tc, tetracycline; Gm, gentamicin.
[b] A 1.1-kbp fragment containing the *nptI* gene from Tn*601* was ligated to *Bam*HI-*Eco*-RI linkers and cloned into the unique *Bam*HI site of pBR322.

oncogenicity on *Kalenchoe daigremontiana* leaves as described by de Framond et al. (14).

Mini-prep DNAs from *E. coli* and *A. tumefaciens*. Small-scale preparations of *E. coli* plasmid DNA were isolated by the rapid alkaline lysis method of Birnboim and Doly (6) as described by Maniatis et al. (44). Small-scale preparations of *Agrobacterium* DNA were prepared from 1.5 ml of an overnight culture grown in L broth with the appropriate antibiotics. Bacteria were pelleted in a microfuge (Brinkmann Instruments, Inc., Vineland, N.J.) and then treated with a modification of the rapid alkaline lysis method as described by Farrand et al. (19). All resuspensions were done with an enlarged Pipetman tip rather than by vortexing to avoid shear damage to the large plasmids.

Enzymes, gel electrophoresis, and DNA blot hybridizations. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or New England BioLabs, Inc. (Beverly, Mass.), and used as recommended by the supplier. Sticky-end ligations were as described by de Framond et al. (14). Sticky-end fragments were rendered blunt and then ligated as described by de Framond et al. (14) except that incubations were done at 22°C for 16 h. Gel electrophoresis and DNA blot hybridizations were as described by Hood et al. (33).

***A. tumefaciens* and *E. coli* transformation.** *E. coli* transformation was performed by the calcium chloride procedure of Cohen et al. (12). *A. tumefaciens* was transformed by the freeze-thaw method of Holsters et al. (31).

Construction of pMO25-E°. We needed a small, easily-manipulated, wide-host-range vector lacking an *Eco*RI site to construct the pTiBo542 helper plasmid pEHA101. The pMO25 plasmid provided by Wayne Barnes at Washington University Medical School is derived from pRK252 (D. Helinski), is 10 kilobases long, and is composed of a P1 incompatibility replication origin, the M13 replication origin, tetracycline resistance gene, and a linker region with unique cloning sites for *Bam*HI, *Eco*RI, *Hin*dIII, *Xba*I, and *Bgl*II. To destroy the *Eco*RI site, 5 μg of plasmid DNA was digested with *Eco*RI, rendered blunt with the large fragment of DNA polymerase I, and religated. Tetracycline-resistant *E. coli* colonies transformed with this DNA were screened by digestion of mini-prep DNA with *Bam*HI or *Eco*RI to confirm retention of the former and loss of the latter sites. One such plasmid was designated pMO25-E°.

Construction of pEHK2 for introduction into A281. Figure 1 illustrates the construction of pEHK2. We used the 2.35-kbp *Bam*HI-*Eco*RI fragment at the left edge of *Bam*HI fragment 5 as the left outside fragment of this T region (see Fig. 3). *Bam*HI fragment 5 contains a left T-DNA border (32a), and even though our outside fragment probably contains this border, we judged that this would not be detrimental. Our greatest concern was to delete T-DNA without deleting any of the virulence genes. This *Bam*HI-*Eco*RI fragment was cloned into *Bam*HI-*Eco*RI-digested pBR328, a digest that left the ampicillin resistance gene on the vector intact. DNA from the resulting clone, pβ108, was digested with *Pst*I and *Bam*HI, ligated to *Pst*I-digested pMO25-E°, and transformed into *E. coli* LE392. The *Pst*I fragment of pβ108 is composed of a 1.7-kbp *Pst*I-*Eco*RI piece of the original T-region fragment plus a 1.1-kbp *Eco*RI-*Pst*I fragment of pBR328. The *Bam*HI digestion was done to inactivate the remaining part of the vector of pβ108 in the ligation reaction. The resultant clones were screened for orientation of the *Pst*I insert relative to the asymmetric *Eco*RI site. Clone pπ1 was chosen for further manipulation because it oriented the *Eco*RI site close to the *Bam*HI site in the linker region of pMO25-E°. Digestion of this clone with *Bam*HI and *Eco*RI would delete the pBR328 sequences.

The right outside fragment of the T-region was an 8-kbp *Eco*RI-*Bam*HI piece on the right side of *Bam*HI fragment 2a (see Fig. 3A). This fragment was cloned into *Bam*HI-*Eco*RI-digested pHC79, a digestion which allowed selection on ampicillin. DNA from clone pδ108 was digested with *Eco*RI and *Bam*HI, ligated to *Eco*RI-*Bam*HI-digested pπ1, and transformed into *E. coli* LE392. We then had a construct, pπδ307, in a wide-host-range vector that contained left and right outside T-region fragments with a unique *Eco*RI site between them (Fig. 1). The kanamycin resistance gene from pMON249 (Fig. 1; Table 2) was inserted into this unique *Eco*RI site, resulting in pEHK2, a tetracycline-resistant, kanamycin-resistant, ampicillin-sensitive clone.

Construction of pEHA101 from pEHK2. Mini-prep DNA of

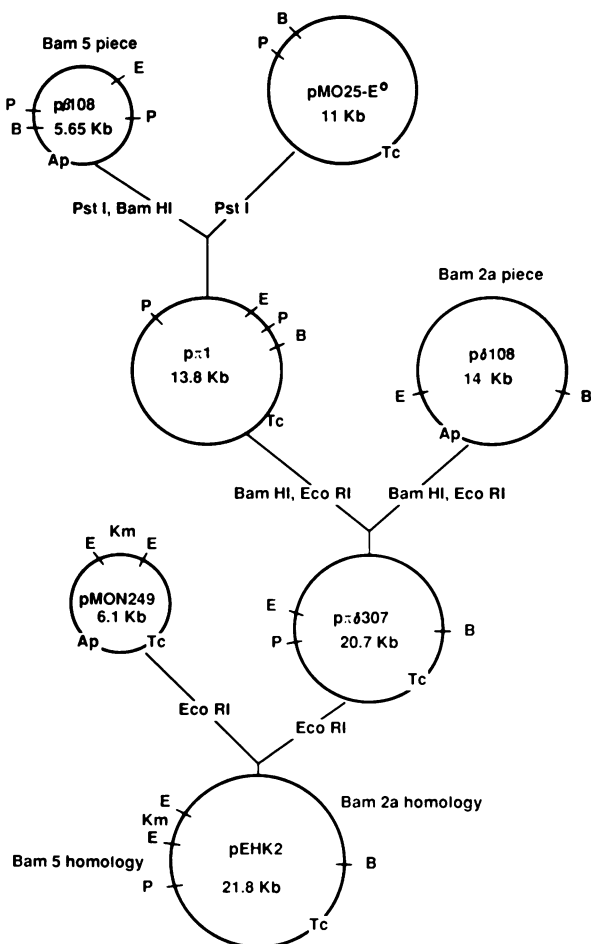

FIG. 1. Schematic representation of the cloning strategy used to construct pEHK2. This plasmid was transformed into strain A281 to effect double-crossover deletion of T-DNA from pTiBo542. The general strategy was to clone fragments from outside the T region (left edge of *Bam*HI-5 and right edge of *Bam*HI-2a) together onto a wide-host-range replicon and place a kanamycin resistance marker between them. See the text for details of construction.

plasmid pEHK2 was transformed into *A. tumefaciens* A281, and selection was made on nutrient agar (Difco Laboratories, Detroit, Mich.) plus kanamycin and nalidixic acid (a chromosomal marker). Colonies were screened for tetracycline resistance, the drug resistance gene present on pMO25-E°. To select for the double crossover in regions of homology between pEHK2 and pTiBo542, the eviction plasmid R751-pMG2 was mated into the pEHK2-containing strain of A281. R751-pMG2 is in the P1 incompatibility group (as is pMO25-E°) and carries a gentamicin resistance gene. When cells are grown on gentamicin plus kanamycin, the pMO25-E° replicon is evicted. A colony selected in this way that was tetracycline sensitive was called EHA100 and contained plasmids pEHA101 and R751-pMG2.

Mini-prep DNA, either undigested or digested with *Bam*HI, from EHA100, A281, and an *E. coli* strain carrying R751-pMG2 was separated on 0.8% agarose gels (Fig. 2A and D). In Fig. 2, the lanes of A281 DNA were from one experiment, whereas the lanes of DNA from EHA100 and R751-pMG2 were from another similar experiment. Southern blots made from these gels were probed with pEHC277, a T-region cosmid. The undigested A281 Ti plasmid band and several bands in the *Bam*HI-digested A281 DNA hybridized to this probe (Fig. 2B and E). However, no sequences from EHA100 or R751-pMG2 DNA hybridized, indicating that these sequences had been deleted from pEHA101 and were not homologous to any sequences in R751-pMG2. Identical blots were probed with pEHC293 (for A281) or pEHC180 (for EHA100 and R751-pMG2), clones from the region of pTiBo542 to the left of T-DNA. In this case, hybridization to the undigested EHA100 Ti plasmid band and several *Bam*HI fragments in the digested EHA100 and A281 DNAs was observed (Fig. 2C and F). Once again, no hybridization to R751-pMG2 sequences was seen. These data confirmed that the deletion of T-DNA was complete.

Before strain EHA100 could be used in complementation experiments, the P1-incompatible R751-pMG2 plasmid had to be removed. The octopine, nopaline, and L,L-succinamopine T-DNAs, pAL1050 (28), mini Ti/pRK (14), and pCS277 (see below), respectively, all have P1 replication origins and thus could not coexist with the eviction plasmid. Strain EHA100 was grown for several generations in L broth plus kanamycin but without gentamicin and then plated on rich medium with kanamycin only. Of 3×10^4 colonies screened, 2 were gentamicin sensitive and were named EHA101 and EHA102. These strains were shown by gel electrophoresis of mini-prep DNA to have lost the R751-pMG2 plasmid. EHA101 was subsequently used in the complementation experiments described in Results.

Plants, growth conditions, and inoculations. Tobacco, *Nicotiana tabacum* L. cv. Havana, and tomato, *Lycopersicon esculentum* Mill. cv Rutgers, plants were greenhouse grown from seed in 4-in. (diameter; 1 in. = 2.54 cm) pots. Inoculations were performed when plants were 6 to 10 in. tall by using log-phase overnight bacterial cultures, the titers of which were estimated by reading the A_{600}. Bacteria were pelleted and resuspended at a density of 5×10^8 cells per 10 µl. Two wounds per plant stem were made with an

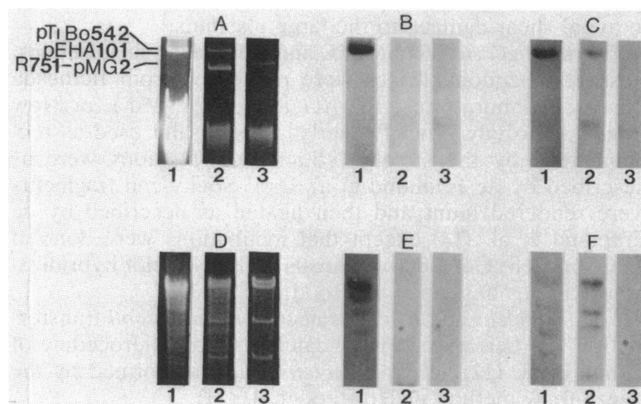

FIG. 2. Verification of the structure of pEHA101. (A) Ethidium bromide-stained gel of DNA prepared from *A. tumefaciens* A281 (lane 1), strain EHA100 (lane 2), or an *E. coli* strain carrying the R751-pMG2 plasmid (lane 3). (B) Southern blots of gels shown in A, probed with a cosmid from the T-DNA region of pTiBo542 (pEHC277 in Fig. 3B). Lanes, as in A. (C) Southern blots as in B, probed with a cosmid from the region to the left of T-DNA from pTiBo542 (pEHC293, lane 1; pEHC180, lanes 2 and 3). (D) Ethidium bromide-stained gel of *Bam*HI-digested DNA prepared from *A. tumefaciens* A281 (lane 1) or EHA100 (lane 2) or the *E. coli* strain carrying the R751-pMG2 plasmid (lane 3). (E) Southern blots of gels in D, probed with pEHC277, the T-DNA probe. Lanes as in D. (F) Southern blot as in E, probed with pEHC293 (lane 1) or pEHC180 (lanes 2 and 3).

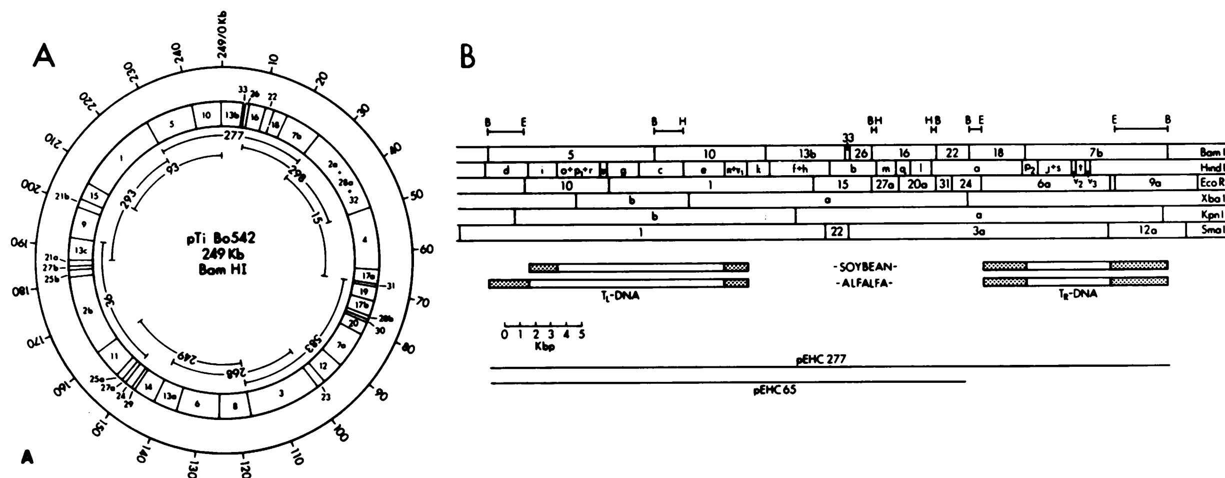

FIG. 3. *Bam*HI restriction endonuclease map of pTiBo542 (A) and a detailed map of the T region (B). The numbers in B represent fragments in a digest of total Ti plasmid with the enzymes indicated. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. The lowercase letters represent T-region fragments in descending order of size. The boxes located directly below the T-region map in B represent T-DNA content in soybean and alfalfa tumor tissues (32a). Bold lines below this same map represent inserts in cosmid clones (pEHC277 and pEHC65) used in *trans* complementation experiments.

18-gauge hypodermic needle, the wound was surrounded with petroleum jelly, and 10 μl of bacteria was applied per wound.

Scoring of tumors. Tumors were subjectively scored according to size as compared with those induced by control strains in each experiment. Tumors were scored on a scale of zero (no tumor) to four (the hypervirulent response typical of A281-incited tumors). Tumors were weighed in some experiments to compare this parameter to their numerical score. Means and standard deviations were calculated for the scores of tumors incited by each strain. In cases in which the standard deviations overlapped, a *t* test for significant differences between the means was done. Photographic records were also kept.

Auxin determinations. *A. tumefaciens* strains were grown overnight in 100 ml of L broth. At the end of the culture period, the bacteria were removed by centrifugation, and 10 mg of butylated hydroxytoluene per liter was added to the medium. Samples were filtered through sterile 0.45-μm (pore size) filters (Schleicher & Schuell, Inc., Keene, N.H.). Twenty-five milliliters of the medium was purified, and [^{14}C] indoleacetic acid (IAA) tracer was added to determine recovery. The solution was titrated to pH 3.0 with acetic acid and filtered through an 0.2-μm filter. Sample auxins were bound to a C18 Sep-pak resin (Waters Associates, Inc., Milford, Mass.) by being passed through a Sep-pak cartridge that had been wet with 5.0 ml of methanol and washed with pH 3.0 acetate buffer (0.01 N acetic acid titrated to pH 3.0 with 0.01 M sodium acetate). Cartridges containing bound auxins were washed with 20 ml of pH 3.0 phosphate buffer–5% methanol (phosphate buffer was distilled water titrated to pH 3.0 with H_3PO_4). IAA was eluted from the Sep-pak with two 5-ml washes of 100% methanol. Volumes of the eluants were reduced on a speedvac (Savant Instruments, Inc., Hicksville, N.Y.) to approximately 200 μl. The sample volume was increased to 2 ml with pH 3.0 phosphate buffer and filtered through a low-dead-volume 0.45-μm filter. The IAA was separated from impurities by preparative reversed-phase high-pressure liquid chromatography and quantitatively analyzed on high-pressure liquid chromatography as described by Hein et al. (25). A Biophase C8 column was used at 45°C (Bioanalytical Systems, West Lafayette, Ind.). IAA concentration was determined by comparing sample peaks with standard peaks and calculating the amount based on recovery of the [^{14}C]IAA tracer added at the beginning of purification.

RESULTS

Construction of *Agrobacterium* strains used to determine the Ti plasmid region responsible for the hypervirulence of strain A281. The pTiBo542 plasmid has been physically characterized (33, 42), and its T-DNA has been defined (32a). Figure 3A displays the *Bam*HI restriction endonuclease map of pTiBo542, as well as a detailed map of its T region (Fig. 3B). The extent of L,L-succinamopine T-DNA in a soybean tumor line and an alfalfa tumor line, with T_L (left portion)-DNA and T_R (right portion)-DNA components, is also shown in Fig. 3B (32a). The lines below this map labeled pEHC277 and pEHC65 illustrate the inserts in two cosmid clones from the T region of pTiBo542 (33) that were used in the virulence assays reported here. pEHC65 contains T_L-DNA sequences only, whereas pEHC277 contains both T_L- and T_R-DNA sequences. The virulence region of pTiBo542 extends from *Bam*HI fragment 2b through *Bam*HI fragment 6 (42).

Our purpose in the experiments reported here was to determine whether the hypervirulence phenotype of strain A281 was a characteristic of the T-DNA or of the virulence region of pTiBo542, the resident Ti plasmid of strain A281. To determine whether this was a characteristic of T-DNA, our strategy was to use the T-region clones described above in *trans* complementation experiments with virulence regions from heterologous Ti plasmids. pHC79 (30) is the cosmid vector used to clone partial digests of pTiBo542 (33). This vector is a derivative of pBR322 and is not capable of replicating in *A. tumefaciens*. Therefore, cointegrates were formed between each cosmid clone and a wide-host-range vector. To this end, each cosmid clone, pEHC277 or pEHC65, was mated into an *E. coli* strain carrying pRK325, a shuttle vector consisting of a wide-host-range plasmid, pRK290, and a 700-base-pair piece of pBR325 in its unique *Eco*RI site (Helmer et al., in preparation). The pRK290

TABLE 3. Plant responses to various *A. tumefaciens* strains

Strain	*vir* region type	T-DNA type	Mean tumor size (±SD) on[a]:	
			Tobacco plants	Tomato plants
Whole Ti plasmid strains				
A281	L,L-SAP	L,L-SAP	3.8 ± 0.8	3.0 ± 0.9
Bo542	L,L-SAP	L,L-SAP	0.5 ± 0.2	0.5 ± 0
A208	NOP	NOP	0.5 ± 0.3	2.0 ± 0.7
Ach5	OCT	OCT	1.9 ± 0.2	1.1 ± 0.4
Bipartite strains				
LBA4434	OCT	OCT	2.0 ± 1.1	1.4 ± 0.7
LBA4404(mini Ti/pRK)	OCT	NOP	0.9 ± 0.5	1.0 ± 0.7
LBA4404(pCS65)	OCT	L,L-SAP	1.1 ± 0.5	0.7 ± 0.4
A479(pAL4404)(pCS65)[b]	OCT	L,L-SAP	2.2 ± 0.4	0.8 ± 0.4
A479(pAL4404)(pCS277)[b]	OCT	L,L-SAP	2.0 ± 0.6	1.6 ± 0.7
ASE-1(mini Ti/pRK)	NOP	NOP	0.1 ± 0.1	0.2 ± 0.1
ASE-1(pCS65)	NOP	L,L-SAP	0.5 ± 0.09	0.3 ± 0.2
EHA101(pCS277)	L,L-SAP	L,L-SAP (L+R)	3.8 ± 0.8	1.7 ± 0.8
EHA101(pCS65)	L,L-SAP	L,L-SAP (L)	2.1 ± 1.2	0.7 ± 0.3
EHA101(pAL1050)	L,L-SAP	OCT	3.6 ± 1.1	2.6 ± 1.4
EHA101(mini Ti/pRK)	L,L-SAP	NOP	0.7 ± 0.3	1.3 ± 0.9
Control strains				
A479	None	None	0	0
A479(pCS277)	None	L,L-SAP	0	0
A479(pAL4404::Tn*903*)	OCT	None	0	0
LBA4404	OCT	None	0	0
ASE-1	NOP	None	0	0
EHA101	L,L-SAP	None	0	0

[a] Numbers are tumor sizes rated on a scale of 0 to 4, 0 meaning no tumor and 4 meaning a hypervirulent A281-type tumor. Correlated tumor weights were as follows: (tobacco) 4, 3.5–5.75 g; 3, 3.0 to 3.5 g; 2, 1.25 to 2.0 g; 1, 0.85 to 1.25 g; 0.5, 0.2 to 0.5g; and (tomato) 4, 3.5 to 4.5 g; 3, 2.0 to 3.0 g; 2, 0.9 to 1.5 g; 1, 0.35 to 0.5 g; 0.5, 0.1 to 0.25 g.

[b] The ::Tn*903* designation on pAL4404 was omitted for ease of reading the table. Abbreviations: L,L-SAP, L,L-succinamopine; OCT, octopine; NOP, nopaline.

origin of replication belongs to the P1 incompatibility group and allows the plasmid to replicate in both *E. coli* and *A. tumefaciens*. The 700-base-pair pBR325 sequence allows cointegration to occur between the shuttle vector and pBR-derived plasmids. Both the cosmid and shuttle vector are maintained in the same *E. coli* cell, and this strain is used to donate cointegrated plasmids to *A. tumefaciens*, with selection being made for stable carbenicillin resistance (carried on the pHC79 vector). The cointegrate plasmids in this work resulting from such a cross are designated by the pCS prefix.

To test the virulence region separately from the T-DNA of pTiBo542 for hypervirulence, a helper plasmid, pEHA101, containing the *vir* region but lacking T-DNA was constructed. The strategy we used to construct such a helper plasmid was to clone a restriction fragment from each side of the T region into a wide-host-range vector (pMO25-E°) and then place an antibiotic resistance marker between them. This clone, pEHK2, was introduced into *A. tumefaciens* A281, and selection was made for a double-crossover event that would yield a T-DNA deletion derivative of pTiBo542 called pEHA101. Construction of pEHK2 and its subsequent manipulations to yield pEHA101 are detailed in Materials and Methods (Fig. 1 and 2).

Tumor responses of plants to various *Agrobacterium* strains. To test the *vir* regions and T-DNAs from various Ti plasmids, we assayed tumor formation on plants by *Agrobacterium* strains harboring combinations of these regions in bipartite strains. Table 1 describes these strains and their relevant characteristics. The three types of Ti plasmids assayed in this bipartite system were pTiBo542, the L,L-succinamopine Ti plasmid on which this work was focused, pTiAch5, an octopine Ti plasmid, and pTiT37, a nopaline Ti plasmid. The strains of *A. tumefaciens* harboring these intact Ti plasmids are A281, Ach5, and A208, respectively (Table 1). Two different chromosomal backgrounds are represented by these strains—A281 and A208 are in the C58-cured chromosomal background (52), and Ach5 is in its wild-type chromosomal background. We attempted to make enough comparisons among these strains to assess the contributions of the different chromosomal backgrounds to tumor formation.

Table 3 describes the responses of tobacco and tomato plants to the bacterial strains used in this work. Numbers assigned to these plant responses are means and standard deviations of tumor sizes from several inoculations on a scale of zero (no tumor formation) to four (the size of hypervirulent tumors incited by strain A281). Two-tailed *t* tests were performed to assess the significance of differences among the means. A range of tumor weights for each rating is given in the table legend.

(i) Wild-type strains. The responses of plants to the wild-type Ti plasmids are given at the top of Table 3. Strain A281 consistently incited the largest tumors on tobacco and tomato plants. The octopine strain Ach5 was second in virulence on tobacco plants, whereas the nopaline strain A208 was second most virulent on tomato plants. Strain Bo542, the wild-type isolate harboring pTiB542, was weakly virulent on both tobacco and tomato plants.

(ii) Bipartite strains involving the octopine *vir* region. Table 3 also presents results of studies on tumors incited by bipartite strains containing the *vir* regions from the three types of Ti plasmids discussed above. For each group of strains involving a different *vir* region, the one reconstructing the wild type is the first strain listed. The reconstructed octopine bipartite strain LBA4434 elicited a tumor response on tobacco and tomato plants very similar to that elicited by its wild-type progenitor Ach 5. This octopine *vir* plasmid, pAL4404, when combined with nopaline T-DNA, incited tobacco tumors similar to those from the wild-type nopaline strain A208. On tomato plants, however, tumor formation by this bipartite strain was significantly less than that of A208 ($P < 0.02$). The pAL4404 *vir* plasmid was tested in both the Ach5 (LBA4404) and C58 (A479) chromosomal backgrounds with the pTiBo542 T_L-DNA plasmid pCS65. Tumors were significantly larger ($P < 0.01$) on tobacco plants from the strain containing the plasmids in the C58 chromosomal background, indicating that chromosomal background had an effect on tumor formation in tobacco plants (Table 3). However, on tomato plants no differences were observed in tumor formation with these plasmids in either of the chromosomal backgrounds. The pCS277 construct (containing T_L- and T_R-DNAs) with the octopine *vir* region incited tumors on tobacco plants similar to those induced by the pCS65 construct. However, on tomato plants, pCS277 tumors were significantly larger ($P < 0.01$). These results indicated that there may be a locus in T_R-DNA that is involved in tumorigenicity on tomato plants. More important, in no instance was the pTiBo542 T-DNA complemented to hypervirulence by the octopine *vir* region on tobacco and tomato plants.

(iii) Bipartite strains involving the nopaline *vir* region. When the nopaline Ti plasmid pTiT37 was separated into a bipartite system as exemplified by strain ASE-1(mini Ti/pRK), tumors incited on tobacco and tomato plants were significantly reduced in size compared with A208-incited tumors ($P < 0.01$) (Table 3). The pTiBo542 T_L-DNA (pCS65), when complemented with the nopaline *vir* region, incited tumors significantly smaller than A281 wild-type-induced tumors on tobacco and tomato plants ($P < 0.01$). These tobacco tumors were also significantly smaller than those incited by the bipartite strain containing this T-DNA with its own L,L-succinamopine *vir* region ($P < 0.01$). The same was true of tomato tumors incited by strain ASE-1(pCS65) ($P < 0.05$). Thus, the nopaline virulence region, like the octopine virulence region, could not complement pTiBo542 T-DNA to hypervirulence on tobacco and tomato plants.

(iv) Bipartite strains involving the L,L-succinamopine *vir* region. The most interesting results from these experiments were the tumor responses of plants to strains constructed with the pTiBo542 *vir* region. Both T-DNA constructs described above, pCS277 and pCS65, were used to reconstruct the pTiBo542 plasmid in the bipartite system. The pCS277 plasmid, with its homologous *vir* region, pEHA101, produced an average wild-type tumor response on tobacco plants (Table 3). However, this strain produced a reduced tumor response on tomato plants. The pCS65 construct incited a reduced tumor response on tobacco and tomato plants compared with A281 or the pCS277-containing bipartite strain. These results suggested that there may be a locus (or loci) that contributes to tumor size on tobacco plants in the T_R-DNA region. These data also suggested that a reduced tumor response on tomato plants is due to having the plasmid separated into a bipartite system and that a locus affecting tomato tumor size may be present in T_R-DNA. Similar results were obtained with these T-DNAs and the octopine *vir* region on tomato plants.

A bipartite strain composed of the L,L-succinamopine *vir* region and the octopine T-DNA, EHA101(pAL1050), was highly virulent on tobacco and tomato plants (Table 3). The tumors incited by this strain were significantly larger than those incited by either the octopine wild-type parent Ach5 or the reconstructed strain LBA4434 ($P < 0.01$). These exciting results indicated that the major determinants for the hypervirulence of strain A281 must lie in the non-T-DNA region of pTiBo542. When nopaline T-DNA was complemented with the L,L-succinamopine *vir* region, tumor size on tobacco plants was not significantly different than the tobacco response to strain A208, but it was significantly increased over the response to nopaline T-DNA in a bipartite strain with its own *vir* region, ASE-1 ($P < 0.01$). EHA101(min Ti/pRK)-incited tobacco tumors were approximately equal to those incited by the octopine *vir* region with nopaline T-DNA. Tumors incited by EHA101(mini Ti/pRK) on tomato plants were not significantly different than those incited by A208 or by a bipartite strain with nopaline T-DNA and the octopine *vir* region. However, they were significantly larger than tomato tumors incited by a bipartite strain containing the nopaline T-DNA complemented with the nopaline *vir* region ($P < 0.01$) (Table 3). Taken together, these results suggested that the pTiBo542 *vir* region plasmid can complement T-DNAs to greater virulence on solanaceous plants than can the nopaline *vir* region. Even though the tumors incited by EHA101(mini Ti/pRK) were not hypervirulent, they were larger than those incited by the homologous bipartite strain containing this nopaline T-DNA.

Table 4 presents a hierarchy of tumor responses of plants to the various bacterial strains used in this work. Figure 4 illustrates some typical tobacco plant responses to strains containing these wild-type Ti plasmids and some of their bipartite derivatives. Differences in stem swelling and tumor size reflected the effects of the different strains on the plants (Fig. 4, legend.) The hypervirulence of strain A281 was clearly visible. Moreover, secondary tumors were noted in the stems above the inoculation sites on tobacco for strains A281 and EHA101(pCS277) (Fig. 4).

Conclusions regarding intact Ti plasmids are that A281 was the most virulent on tobacco and tomato plants and Bo542 was the least virulent. The L,L-succinamopine and octopine T-DNAs were most tumorigenic when complemented with the L,L-succinamopine *vir* region on tobacco and tomato plants (Table 4). However, there were several cases in which octopine *vir* region complementation of L,L-succinamopine T-DNAs was equal to that of the homologous *vir* region. In these cases, tumor size equaled that observed from wild-type octopine strains but was less than the hypervirulent tumor size obtained from strain A281 (Table 3; Fig. 4). Responses of plants to strains with L,L-succinamopine and nopaline T-DNAs complemented with the nopaline *vir* region were significantly less than responses to their wild-type progenitor strains (Table 3; Fig. 4), L,L-succinamopine and octopine T-DNAs were complemented to similar levels of hypervirulence on tobacco plants by the L,L-succinamopine *vir* region. On tomato plants, octopine T-DNA was better complemented than the homologous L,L-succinamopine T-DNA by the L,L-succinamopine *vir* region (Table 3). Tumors incited by the bipartite strain EHA101(pAL1050) were much larger on tobacco and tomato plants than those incited by Ach5, the parental strain of this T-DNA. These data strongly suggested that the primary loci

TABLE 4. Hierarchy of tumor formation on each plant assayed as a function of Ti plasmid or T-DNA type in bipartite strains

Plasmid or T-DNA type and plant	Strains or Complementing *vir* region types showing the: Greatest response ⟶ Least response
Whole Ti plasmid	
Tobacco	A281 > Ach5 > A208 =[a] Bo542
Tomato	A281 > A208 > Ach5 > Bo542
Octopine T-DNA	
Tobacco	L,L-SAP > OCT[b]
Tomato	L,L-SAP > OCT[b,c]
Nopaline T-DNA	
Tobacco	L,L-SAP = OCT[b] >> NOP
Tomato	L,L-SAP = OCT[b] > NOP
L,L-Succinamopine T_L- + T_R-DNA	
Tobacco	L,L-SAP > OCT[d]
Tomato	L,L-SAP = OCT[d]
L,L-Succinamopine T_L-DNA	
Tobacco	L,L-SAP = OCT[d] > OCT[b] > NOP
Tomato	L,L-SAP = OCT[d] = OCT[b] > NOP[c]

[a] =, Means were not significantly different.
[b] Ach5 chromosomal background; i.e., LBA4404.
[c] Means were significantly different at $P < 0.05$.
[d] C58 chromosomal background; i.e., A479(pAL4404::Tn*903*).

(locus) responsible for the hypervirulence of strain A281 are sequences on pTiBo542 outside the T-DNA region.

In an effort to understand the mechanism of hypervirulence, we investigated auxin levels in culture filtrates of A281, Bo542, A208, and A479. We found 0.14 μg of auxin per ml in culture filtrates of A479, the Ti plasmidless strain, but no measurable levels of auxin in culture filtrates of the other strains (data not shown). These results suggested that auxin production by the bacterium at the time of inoculation onto plants probably is not involved in the hypervirulence of strain A281.

DISCUSSION

The T and *vir* regions are the major regions of the Ti plasmid of *A. tumefaciens* that are responsible for oncogenicity on plants. Each of these regions has been defined genetically through transposon mutagenesis and complementation experiments. In the studies reported here we assessed the contribution of each of these regions from pTiBo542 to the hypervirulence of strain A281.

Conclusions that may be drawn from the data presented above are as follows. (i) The T-DNA of strain A281 is not hypervirulent on solanaceous plants when complemented in *trans* with either the octopine or the nopaline *vir* region. (ii) The L,L-succinamopine helper plasmid pEHA101, whose construction is reported here, can complement its own T-DNA to hypervirulence on tobacco plants and octopine T-DNA to hypervirulence on tobacco and tomato plants.

The bipartite system that we used here to test the hypervirulence of strain A281 had been shown to work previously by de Framond et al. (14) and Hoekema et al. (28). We used this concept in our experiments to study the interaction of T and *vir* regions from several Ti plasmids. Our results extend the concept of functional homology of virulence systems (29) to include the *vir* region of an L,L-succinamopine plasmid. Other L,L-succinamopine strains likely to be included in this general concept are AT1TF and A543 (11).

The bioassays used here to assess contributions of regions of the Ti plasmid to hypervirulence are difficult to score. Visual examination of tumors appeared to be as reliable a way to order and score these tumors as was recording tumor weight or length and width of tumors. Each set of plants inoculated was uniform within itself but could not be directly compared in terms of stem size or weight with other sets of plants grown at different times of the year. Therefore, even though tumor size was scored subjectively, we found that scaling the tumors from zero to four was the most consistent method. In an effort to determine whether the differences we observed were significant, we performed two-tailed *t* tests on all comparisons discussed in Results.

The C58 chromosome has been shown to contain two virulence operons (16). Other agrobacterial strains are likely to have similar chromosomal regions. Our results showed a minimal effect of chromosomal background on tumor formation on tomato plants but a significant effect on tobacco plants (Table 3). We were concerned that the small tumors we observed from LBA4404(pCS65) might be due to a difference in chromosomal background between this strain and A281. However, even though pTiBo542 T-DNA was more tumorigenic on tobacco plants when complemented with the octopine *vir* region in the C58 chromosomal background than in the Ach5 background, the difference did not account for the lack of hypervirulence [compare strains LBA4404(pCS65) and A479(pAL4404::Tn*903*)(pCS65) in Table 3].

pTiBo542 has structural similarity to the octopine Ti plasmids (32a, 42). The *vir* region genes from this plasmid are efficient at complementing octopine T-DNA, indicating that these two heterologous Ti plasmid regions are functionally quite compatible. The poor interaction of octopine and L,L-succinamopine regions with complementary regions from pTiT37 may be because of small differences in border

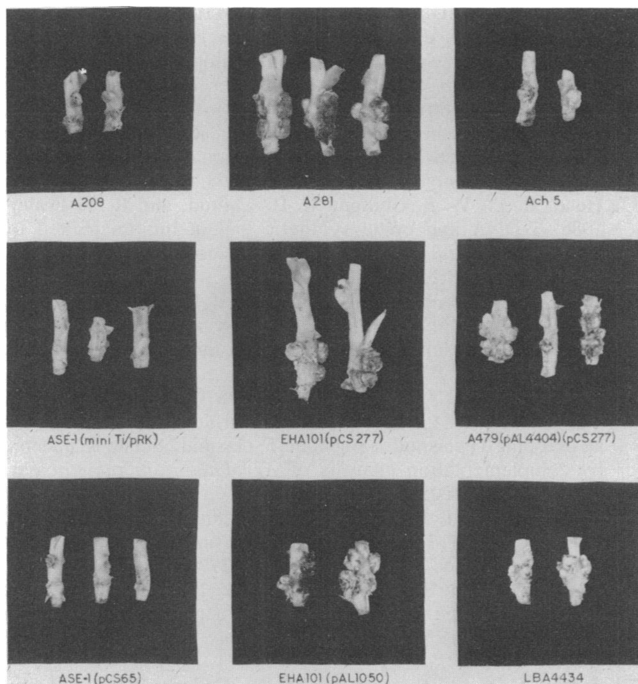

FIG. 4. Tumors incited on tobacco plants by various strains of *Agrobacterium* that were excised for photography. All photographs were taken and printed with the same enlargements so that any differences in stem segment length or width is a function of tumor formation by the strain indicated. A208, A281, and Ach 5 are nopaline, L,L-succinamopine, and octopine strains, respectively. Strains ASE-1(mini Ti/pRK), EHA101(pCS277), and LBA4434 are bipartite reconstructions of these same strains. Note the hypervirulent tumors incited by strains A281, EHA101(pCS277), and EHA101(pAL1050). See the text and Table 3 for more detail.

or adjoining sequences that cut down on the efficiency of T-DNA transfer. A way to address this question would be to put borders from these different Ti plasmids onto otherwise equivalent T-DNAs in bipartite systems and test for tumor formation with the different *vir* regions.

Otten et al. (49) have recently identified a fragment from pTiB6S3 that increases transient nopaline expression in *Kalenchoe* stem tumors incited by nopaline strain C58. This 5-kbp piece of DNA is presumed to contain the *virF* locus as defined by Hooykaas et al. (34) and appears to encode a diffusible factor active either extracellularly (to the bacterium) or in the plant cell. We showed that the hypervirulence of strain A281 is due to non-T-DNA sequences on this Ti plasmid and may act through a mechanism similar to the one described by Otten et al. (49). However, although we have referred to pEHA101 as a *vir* region plasmid, the effect of the hypervirulence has not been shown to be in the *vir* region per se. Therefore, it is premature to conclude from the experiments reported here that the *vir* region itself is responsible for the hypervirulence phenotype of strain A281.

There could be a number of explanations why pTiBo542 is hypervirulent on solanaceous plants. T-DNA copy number per cell could be high; the number of wounded plant cells initially transformed could be high; the timing of transformation could be earlier than for other strains, giving the tumor a head start; or the production of hormones by the bacterium before it comes in contact with the plant could be high. Auxin levels in culture filtrates of A281, Bo542, A208, and A479 were investigated. We found high levels of auxin (0.14 μg/ml) in culture filtrates of A479, the Ti plasmidless strain, but no measurable levels of auxin in culture filtrates of the other strains. These results suggested that bacterial production of auxins is probably not involved in the hypervirulence of A281. The other possibilities could be addressed by using an in vitro transformation system with nononcogenic T-DNAs and the EHA101 helper. An et al. (2) saw a significant difference in transformation frequency by using the whole pTiBo542 plasmid as a helper. Further experiments to define the mechanism of hypervirulence will prove to be interesting and useful.

Technology for plant genetic engineering is rapidly being developed. Vectors exhibiting either a single disarmed Ti plasmid system (*cis*) (20) or a binary-vector strategy (2, 4, 13) have been used to successfully transform plant cells. The pAL4404 octopine *vir* region plasmid has been used in many binary systems to transform plants. We showed that the pTiBo542 helper plasmid is capable of inciting hypervirulent tumors on tobacco and tomato plants with homologous and heterologous T-DNAs. These exciting results should contribute to the progress of plant transformation systems.

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