Genes Responsible for the Supervirulence Phenotype of *Agrobacterium tumefaciens* A281

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Agrobacterium tumefaciens **A281 induces large, rapidly appearing tumors on a variety of plants and has a wider host range than other strains of *A. tumefaciens*. By using Tn3HoHo1 transposon mutagenesis and complementation analysis, a 2.5-kilobase DNA fragment which is responsible for the supervirulence phenotype was identified in the virulence (*vir*) region of the Ti plasmid. This fragment contains the *virG* locus, as well as the 3′ end of the *virB* operon. A clone of this fragment conferred the supervirulence phenotype on A348, a nonsupervirulent strain. The increased virulence was correlated with an increased expression of *vir* genes, which could be achieved by introducing an extra copy of the transcriptional activator *virG* or the supervirulence region for maximum virulence. The virulence of the supervirulent strain A281 could be increased even further if the entire *virB* operon was added in addition to the *virG* operon. A plasmid, pToK47, containing *virB* and *virG* increased the virulence of all *A. tumefaciens* strains into which the plasmid was introduced. These data suggest that a highly virulent binary vector system can be constructed which might prove especially useful in the transformation of certain higher plants.**

Agrobacterium tumefaciens induces tumors on wounded plants by introducing a specific DNA fragment (T-DNA) of the tumor-inducing plasmid (Ti plasmid) harbored by virulent strains of *A. tumefaciens* (4, 17) into plant cells. The uncontrolled expression of the plant growth regulator genes, those for auxin and cytokinin, coded by the T-DNA (1, 22, 27) which becomes integrated into the nuclear DNA of the plant, leads to tumor formation. Another region of the Ti plasmid, the virulence (*vir*) region, encodes most of the functions necessary for T-DNA transfer (13, 14). In the well-studied Ti plasmid pTiA6, six transcriptional units have been identified in the *vir* region (25). Among the *vir* genes whose functions are known, a portion of the *virD* operon encodes for a site-specific endonuclease activity which is involved in T-DNA processing (32), and *virA* and *virG* are involved in the transcriptional regulation of all of the other *vir* genes (26, 30). The functions of the remaining *vir* genes are unknown.

Tumor formation by *A. tumefaciens* is an example of a naturally occurring genetic engineering system, and great efforts have been expended to improve this system as a means of introducing genes of interest into higher plants. *A. tumefaciens* strains vary in their host range, as well as in the time at which tumors appear after infection (21). In this regard, *A. tumefaciens* A281 carrying the plasmid pTiBo542 is of particular interest because of its ability to induce large, early-appearing tumors on a wider range of plants than do other *A. tumefaciens* strains (9, 10, 19). The physical and functional organization of pTiBo542 is highly homologous to that of the well-studied plasmid pTiA6 (16). By using a binary vector system, An (2) has shown that pTiBo542, when used as a helper plasmid gene, gave a transformation efficiency 10-fold-higher than that of other Ti plasmids. This property is associated with the pTiBo542 plasmid because *A. tumefaciens* A348, C58, and A208, carrying Ti plasmids

pTiA6, pTiC58, and pTiT37, respectively, and with the same chromosomal background as strain A136 (7, 19, 28), are not as highly virulent as strain A281. Recently, Hood et al. have reported that a non-T-DNA region of the Ti plasmid is involved in this supervirulence phenotype (10). Here we present results showing that a 2.5-kilobase (kb) region of plasmid pTiBo542 containing *virG* and the 3′ end of *virB* is responsible for the supervirulence phenotype of A281 and that this fragment can confer the supervirulence phenotype on A348. The virulence of the supervirulent strain A281 could be increased further by adding a copy of the entire *virB* operon in addition to the super *vir* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA handling. The *A. tumefaciens* strains used in this study are listed in Table 1. pTiA6 and pTiBo542 cosmid clone banks were constructed in *Escherichia coli* HB101 (15, 16); *E. coli* LE392 was used as a host for subcloning of DNA fragments and plasmid constructions. Recombinant DNA constructs were performed by standard procedures (18).

Infection of intact plants with *A. tumefaciens*. *Glycine max* cultivars (seeds were obtained from D. L. Owens), *Kalanchoe daigremontiana*, *Lycopersicum esculentum*, and *Pisum sativum* were grown in soil in 12-cm pots for 1 to 2 months. The stems were wounded with 18-gauge needles and inoculated with *A. tumefaciens* cells grown on AB minimal agar plates (3) for 2 days at 30°C. The wounds were wrapped with Parafilm after infection. Wounding and inoculation of *K. daigremontiana* leaves were done at the same time with toothpicks.

Nicotiana glauca, *Nicotiana rustica*, and *Nicotiana tabacum* v. Xanthi were grown under sterile conditions on Murashige-Skoog (MS) (20) agar media. The stems or leaves were cut and then inoculated with *A. tumefaciens*.

Preparation of roots and tuber disks and cocultivation with *A. tumefaciens*. The roots of *Daucas carrota* and tubers of *Helianthus tuberosus* and *Solanum tuberosum* were obtained from a grocery store. Their surfaces were sterilized by

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TABLE 1. *A. tumefaciens* strain used

Strain	Ti plasmid	Chromosomal background	Characteristics	Reference
A348	pTiA6NC	C58	Octopine producing, unorganized tumor	8
A281	pTiBo542	C58	L, L-Succinoamopine producing, unorganized tumor	9, 10
A208	pTiT37	C58	Nopaline producing, teratoma	23
A136	None	C58		23
T37	pTiT37	T37	Nopaline producing, teratoma	23
R1000	pRiA4b	A4T	Octopine producing, hairy root tumor	29

being soaked in 30% bleach for 5 to 10 min, dipped in 70% ethanol briefly, and then washed in sterile water. Core samples of root and tuber tissues were prepared by using a cork borer (6-mm diameter) and then cutting the cores into disks 1.5 mm thick with a razor blade. The disks were dipped into 2 to 3 ml of an overnight culture of *A. tumefaciens* grown in MG/L broth (28), incubated on MS agar plates for 2 to 3 days, and then transferred to the same media containing 100 μg of carbenicillin per ml. Tumors were evaluated 4 weeks after inoculation.

Preparation of leaf disks and cocultivation with *A. tumefaciens*. Leaf disks were prepared by the method of Horsch et al. (12). Bacteria were grown on AB plates for 2 days, and a loopful of bacterial cells was suspended in 1 ml of MG/L broth for subsequent infection of leaf disks. Agrobacteria on leaf disks were killed with cefotaxime instead of carbenicillin because of the phytohormonelike effect of carbenicillin on *N. glauca* leaf disks.

Introduction of plasmids into *A. tumefaciens*. Three bacterial strains, a donor *E. coli* strain (either HB101 or LE392) carrying the plasmid of interest, a recipient *A. tumefaciens* strain (either A281 or A348), and a conjugal helper *E. coli* strain (HB101) carrying pRK2073 (6) were mated on a nutrient agar plate. The exconjugants were selected on AB agar plates containing the proper antibiotics for the plasmid of interest (kanamycin at 100 μg/ml or carbenicillin at 100 μg/ml). The introduction of plasmids was confirmed by reintroducing the plasmids from *A. tumefaciens* into LE392, followed by DNA preparations and restriction digestions.

Tn3HoHo1 mutagenesis. pTVK85 and pTVK79 were mutagenized with Tn3HoHo1 by the method of Stachel et al. (24). Insertions were mapped after BamHI and EcoRI restriction digestions.

Dot blot assay. RNA samples from *A. tumefaciens* cells were isolated by the method described by Stachel and Zambryski (26). Probes were prepared and hybridized as described by Maniatis et al. (18).

β-Galactosidase assay. For *vir* gene induction, *A. tumefaciens* cells were cultured in MS.2 plus 0.2 mg of 2.4-D liquid medium per liter (2) with 100 μM acetosyringone. Both *vir* gene induction and β-galactosidase assay were performed as described previously (24). All assays were done at least three times on independent cultures with similar results.

RESULTS

Assay system for comparison of virulence. In a search for a rapid, simple way of testing the virulence levels of *A. tumefaciens* strains, 10 species of intact plants and 5 species of cultivars and explants were infected in various ways with *A. tumefaciens* A281 and A348 (Table 2).

Tumors incited by strain A281 appeared 1 to 2 weeks earlier than those incited by A348 on intact plants of seven species: *G. max* cv. Peking (soybean), *K. daigremontiana*, *L. esculentum* (tomato), *N. glauca*, *N. rustica*, *N. tabacum*

(tobacco), and *P. sativum* (pea). No significant difference in the speed of tumor growth was observed, and as a result, after 3 to 4 weeks, the tumors incited by A281 on these plants were larger than those incited by A348 (Fig. 1). In addition, one plant, *G. max* cv. Beeson, was susceptible to infection by A281 but not by A348 (Fig. 1), indicating that A281 has a wider host range than that of A348. Strains A281 and A348 did not show any obvious differences in their ability to infect *G. max* cv. Biloxi and *G. max* cv. Seneca under the experimental conditions used in this study (data not shown). A281 incited a greater number of tumors than did A348 on explants of five species, including *D. carrota* (carrot), *H. tuberosus* (Jerusalem artichoke), *N. glauca*, *P. hybrida*, and *S. tuberosum* (potato).

The responses of leaf disks of *N. glauca* to strains A281 and A348 were distinctly different (Fig. 2) and reproducible. These differences were clear 1 to 2 weeks after cocultivation and infection of five or six randomly chosen leaf disks conveniently controlled for any variation between indiviudal disks. For these reasons, *N. glauca* leaf disks were selected as the primary material for virulence assays in this study.

Experimental strategy to identify supervirulence determinants. Since A281 and A348 have the same chromosomal background and cryptic plasmids, the factor(s) for any difference in virulence must reside on their Ti plasmids, pTiBo542 and pTiA6. This factor(s) could be either some positive factor(s) which is present on pTiBo542 and absent from pTiA6 or some negative factor(s) which is present on pTiA6 and absent from pTiBo542. If the former is the case, the introduction of appropriate fragments of pTiBo542 into A348 should make A348 supervirulent. On the other hand, if

TABLE 2. Response of plant species to infection with A281 and A348

Species	Part of plant infected	Response to[a]:	
		A348	A281
Daucus carrota	Root disk	+	+++
Glycine max cv. Beeson	Stem	−	+
G. max cv. Biloxi	Stem	+	+
G. max cv. Pecking	Stem	+	+++
G. max cv. Seneca	Stem	−	−
Helianthus tuberosus	Tuber disk	+	+++
Kalanchoe daigremontiana	Leaf	+	+++
Lycopersicum esculentum	Stem	+	+++
Nicotiana glauca	Leaf	+	+++
	Stem	+	+++
	Leaf disk	+	+++
N. rustica	Stem	+	+++
N. tobacum cv. Xanthi	Leaf	+	+++
	Stem	+	+++
Petunia hybrida	Leaf disk	+	+++
Pisum sativum	Stem	+	+++
Solanum tuberosum	Tuber disk	+	+++

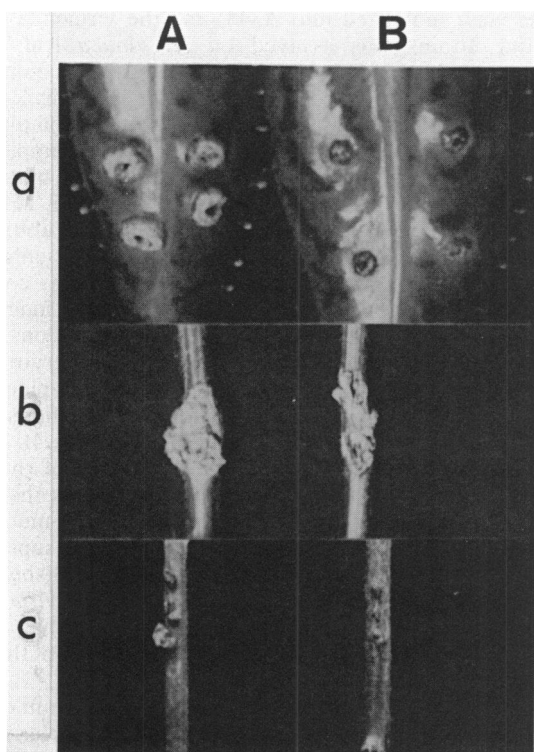[a] +, Tumors formed; +++, massive tumors formed; −, no tumors formed.

FIG. 1. Crown gall tumors incited by A281 (A) and A348 (B) on leaves of *K. daigremontiana* (a) and stems of *G. max* cv. Pecking (b) and cv. Beeson (c). Pictures were taken 2 weeks (a) and 5 weeks (b and c) after inoculation.

TABLE 3. Response of *N. glauca* leaf disks to *A. tumefaciens* strains

Strain	Response to cocultivation of[a]:		Strain	Response to cocultivation of:	
	2 h	24 h		2 h	24 h
A348	−	+	A348(pToK9)		+++
			A348(pSG638)		++
A281	−	+++	A348(pSG637)		++
			A348(pSW163)		+
A136	−		A348(pToK41)		++
A348(pTVK16)		+	A281(pVCK210)		+
A348(pTVK19)		+	A281(pVCK213)		+
A348(pTVK25)		+	A281(pVCK200)		+++
A348(pTVK28)		+	A281(pVCK206)		+++
A348(pTVK34)		+	A281(pVCK217)		+++
A348(pTVK53)		+	A281(pVCK224)		+++
A348(pTVK56)		+	A281(pVCK225)		+++
A348(pTVK66)		+	A281(pVCK230)		+++
A348(pTVK83)		+	A281(pVCK247)		+++
A348(pTVK86)		+	A281(pVCK257)	−	+++
A348(pTVK95)		+	A281(pVCK265)		+++
A348(pTVK177)		+			
A348(pTVK217)		+	A281(pToK9)	−	+++
A348(pTVK320)		+	A281(pTVK178)	−	+++
A348(pTVK323)		+	A281(pTVK217)	−	+++
A348(pTVK347)		+	A281(pTVK320)	−	+++
A348(pTVK349)		+	A281(pTVK85)	+	++++
A348(pTVK79)		+++	A281(pTVK255)	+	++++
A348(pTVK85)		+++	A281(pTVK291)	+	++++
A348(pTVK178)		+++			
A348(pTVK232)		+++			
A348(pTVK255)		+++			
A348(pTVK291)	−	+++			
A348(pVCK224)		+			
A348(pVCK257)	−	++			

[a] ++++, Massive tumors formed all over the edges of disks within 1 week; +++, massive tumors formed all over the edges of disks within 2 weeks; ++, intermediate between + and +++; +, a few separate tumors formed on the edges of the leaf disks; −, no tumors formed.

the latter is the case, the introduction of certain fragments of pTiA6 into A281 should reduce the virulence of A281 to the level seen for A348.

Effect of clones of pTiBo542 on A348. Twenty-three clones in the group pTVK16 to pTVK349 (Table 3) were chosen from a pTiBo542 bank cloned in the wide-host-range vector, pVK102 (16). These clones overlap by at least 6.2 kb with the respective adjacent clones and cover the entire pTiBo542 plasmid. Clones were mobilized into A348, and the virulence of the resulting strain was examined on *N. glauca* leaf disks. Six clones, pTVK79, pTVK85, pTVK178, pTVK232, pTVK255, and pTVK291, enhanced the virulence of A348 to the level seen for A281 (Table 3). Other clones had no significant effect on virulence. All of the supervirulence-

conferring clones came from the virulence region of pTiBo542 and are diagrammed in Fig. 3. They all had *Sal*I fragments 10 and 15a, suggesting that the supervirulence determinants were located within this 9-kb region.

As controls, two clones, pVK224 and pVK257 from the virulence region of pTiA6 (15), were mobilized into A348 and then tested on *N. glauca* as described above. Plasmid
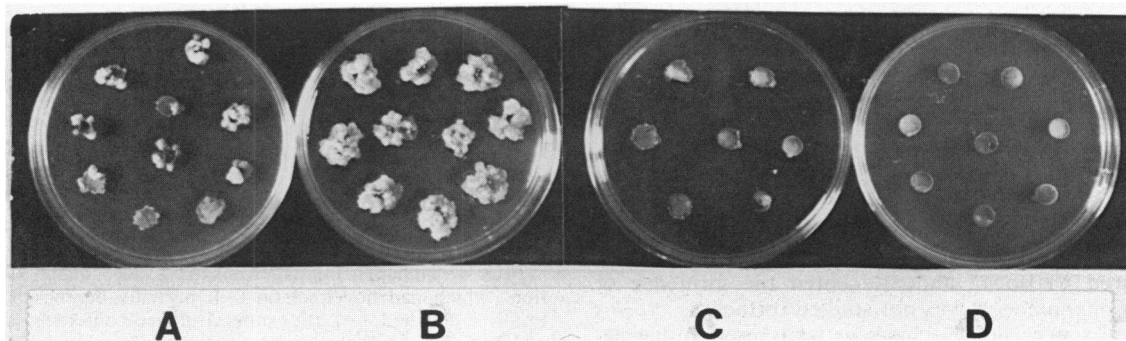

FIG. 2. Tumors formed on *N. glauca* leaf disks by 348 (A), A281 (B), A281::(pTVK85M1::Tn*3*HoHo1) (C), and A136 (D). Leaf disks were cocultivated with agrobacteria for 24 h on standard nurse culture medium (12) and transformed on MS medium which contained 250 μg of cefatoxime per ml. Pictures were taken 3 weeks after inoculation.
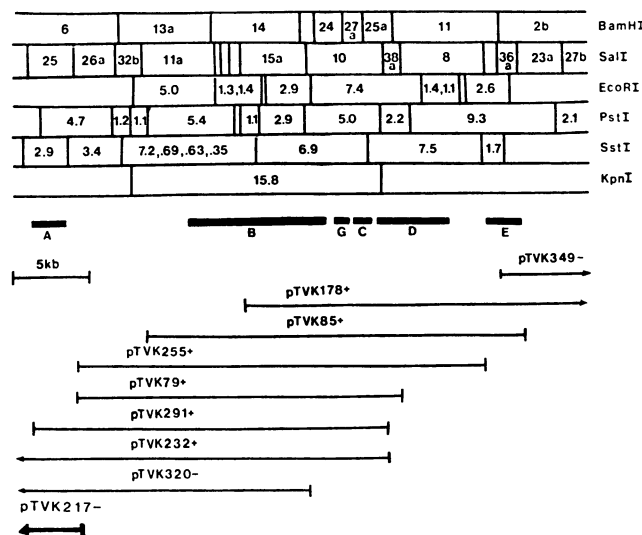
FIG. 3. Clones of pTiBo542 which enhanced (+) or did not enhance (−) the virulence of A348. The clones are shown below the map of the virulence region of pTiBo542. Sizes of fragments (in kilobases) are given on the EcoRI, PstI, SstI, and KpnI maps. A, virA; B, virB; G, virG; C, virC; D, virD; E, virE.

pVK224 carrying the virG, virC, virD, and virE genes of pTiA6 had no significant effect, but pVK257 carrying virA, virB, virG, and virC slightly enhanced the virulence of A348 (Table 3). These data suggest that one or more vir genes contained in pVK257 limit the virulence of A348 and that increasing the copy number of this region increases the virulence, but far less than the effect caused by the pTiBo542-derived cosmid.

Effect of the supervirulence clones on the host range of A348. The ability of the clones of pTiBo542 to enhance the virulence of A348 was tested on another plant species, G. max cv. Beeson, which is susceptible to A281 but not to A348. Strains A348(pTVK178) and A348(pTVK291) incited tumors on G. max cv. Beeson, indicating that the supervirulence determinants identified by using N. glauca leaf disks also extended the host range of A348. On the other hand, pVK257, which had a slightly positive effect on the virulence of A348 on N. glauca leaf disks, could not make A348 virulent on G. max cv. Beeson.

Effect of clones of pTiA6 on strain A281. To determine whether any genes of pTiA6 might be limiting virulence, 11 clones from the group pVCK200 to pVCK265 were chosen from the pTiA6 clone bank (Table 3). These clones, which overlap by at least 6.1 kb with the respective adjacent clones, cover the entire plasmid pTiA6. Clones were mobilized into A281, and the resulting strains were tested on N. glauca leaf disks. Most of the clones had no significant effect on the virulence of A281. However, pVCK210 and pVCK 213 attenuated the virulence of A281 to the level seen for A348. Both clones contain the plasmid incompatibility region of pTiA6, which is reported to be closely related to the incompatibility region of pTiBo542 (16). Therefore, it is possible that the interaction between these incompatibility regions cured pTiBo542 and attenuated the virulence of A281. This phenomenon was not studied further.

Analysis of supervirulence clones by transposon mutagenesis. Cosmids pTVK79 and pTVK85, which made A348 supervirulent, were mutagenized by Tn3HoHo1 (24). Twenty-four insertional mutations were mapped (Fig. 4). Mutant

clones were mobilized into A348, and the virulence of the resulting strains was assayed on N. glauca leaf disks. Insertions M3, M4, M5, M6, M8, M9, and M11 completely abolished the ability of pTVK85 to confer supervirulence to A348. None of these mutations were able to complement virG mutants of A348 in trans, but they did complement virB, virC, and virD mutants, suggesting that these mutations were all in the virG region. Insertions M22, M35, M1, and M7 decreased the supervirulence-conferring ability of pTVK85 and pTVK79, but these mutations still enhanced the virulence of A348 somewhat. Those mutations complemented virG but not the virB mutants of A348, suggesting that M22, M35, M1, and M7 are virB mutations. The remaining 14 insertions did not affect the supervirulence phenotypes of pTVK85 and pTVK79. From complementation assays and restriction mapping, these insertions were mapped to virC, virD, and the 5′ half of virB (Fig. 4). These results suggest that virC, virD, and the 5′ half of the virB operon are not involved in the supervirulence but that virG and the 3′ half of virB operon are involved. Since mutations in the 3′ half of the virB only partially reduced supervirulence and mutations in virG completely abolished the supervirulence phenotype, we conclude that the virG locus alone can enhance virulence on A348 but that for a supervirulence response, both virG and the 3′ half of the virB operon are needed.

To study the effect of the mutation on virulence in cis, all mutations were marker exchanged into the Ti plasmid of A281. All became avirulent except for strains carrying M1, M7, M14, and M31, which became highly attenuated (Fig. 2). The two strains, A281 virBM1::Tn3HoHo1 and A281 virB M7::Tn3HoHo1, could be complemented to supervirulence by introducing one of the following mutated pTVK85 plasmids: M3, M4, M5, M6, M8, M9, or M11. Since these mutations all map to the virG operon, M1 and M7 must be in the virB complementation group.

Identification of the smallest region conferring the supervir-
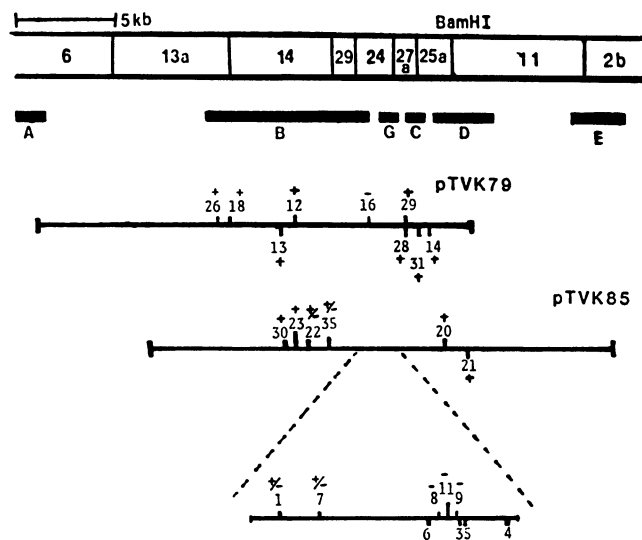
FIG. 4. Tn3HoHo1 mutagenesis to pTVK85 and pTVK79. Insertions which had no effect on (+), partially decreased (+/−), or totally abolished (−) the supervirulence-conferring abilities of pTVK85 and pTVK79 are shown below the BamHI map of virulence region of pTiBo542. The lacZ gene is transcribed from left to right in the insertions whose numbers are given above the line and vice versa.
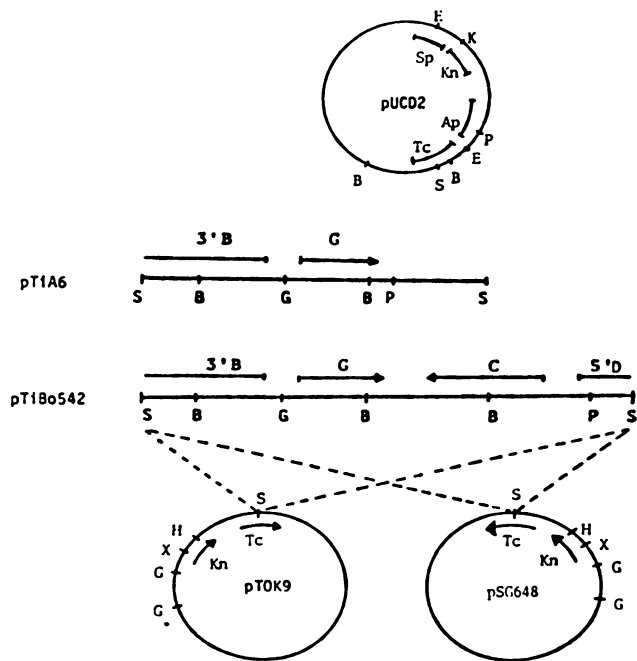
FIG. 5. Plasmid construction. *Sal*I fragment 10 was introduced into pVCK102 in both orientations, yielding pToK9 and pSG648. Also, *Bgl*II-*Pst*I fragments from both pTiA6 and pTiBo542 were introduced into the *Bam*HI-*Pst*I site of pUCD2 to yield pSW163 and pSG637, respectively. Abbreviations: B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I.

ulence phenotype. *Sal*I fragment 10 of pTiBo542 was cloned into the *Sal*I site of pVK102 in two orientations to generate pToK9 and pSG648. *Bam*HI fragment 24 was cloned into the *Bgl*II site of pVK102 to generate pToK41 (Fig. 5). These
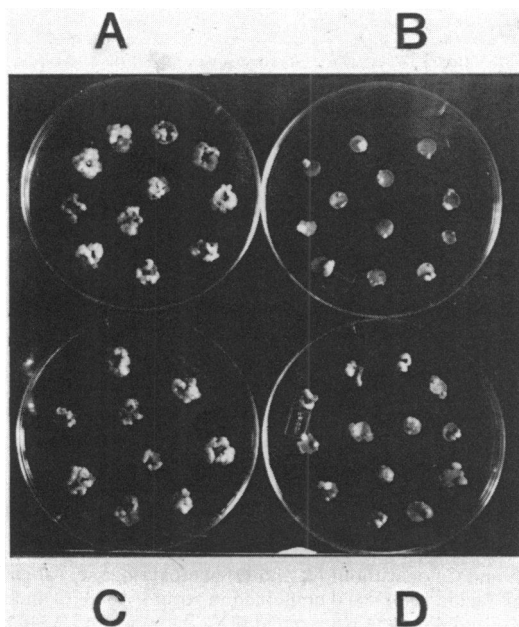
clones were mobilized into A348 and tested for their ability to confer supervirulence. pToK9 conferred supervirulence on A348, whereas pSG648 and pToK41 gave an intermediate response (Fig. 6). The different effects of pToK9 and pSG648 can be explained if the 3' end of *virB*, which is necessary for supervirulence, is transcribed from the upstream Tc^r promoter in pToK9. In the case of pToK41, the truncated *virG* product may have some positive effect on virulence, although it could not complement the *virG* mutant (data not shown). These results indicate that the 3' end of *virB* contained in *Sal*I fragment 10, together with *virG*, is sufficient to give the supervirulence phenotype in A348.

virG gene is expressed at a higher level in A281 than in A348. Small *Bgl*II-*Pst*I fragments containing only *virG* from both pTiA6 and pTiBo542 were subcloned into the *Bam*HI-*Pst*I site of pUCD2 (5), giving pSW163 and pSG637, respectively (Fig. 5). The introduction of these clones into *virG* mutants of A281 showed that pSG637 conferred full supervirulence, while pSW163 gave only normal virulence (Table 3). Furthermore, pSG637 in A348 enhanced the virulence of A348 to an intermediate level, while pSW163 gave no enhancement. Complementation tests showed that pSG637 contains *virC*, in addition to *virG* (data not shown), but from other data presented in this paper, it is clear that *virC* is not involved in the enhancement of virulence. Therefore, we conclude that the meaningful difference between pSG637 and pSW163 is *virG* and that the difference is likely to be quantitative since the two *virG* genes are highly homologous in their sequences (data not shown). This pre-
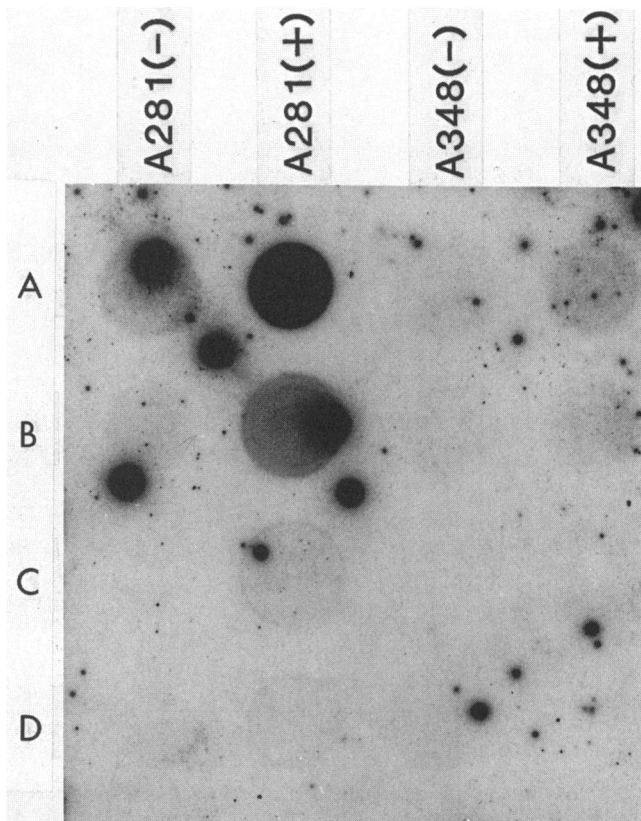

FIG. 6. Tumors formed on *N. glauca* leaf disks 2 weeks after inoculation by A281 (A), A348 (B), A348(pToK9) (C), and A348 (pSG648) (D).

FIG. 7. Dot blot assay. The same amounts of RNA prepared from A348 and A281, with (+) and without (−) induction for 16 h, were blotted onto nitrocellulose paper and probed with ^{32}P-labeled pSW163 DNA. (A) 1 μg; (B) 0.5 μg; (C) 0.1 μg; (D) 0.05 μg.
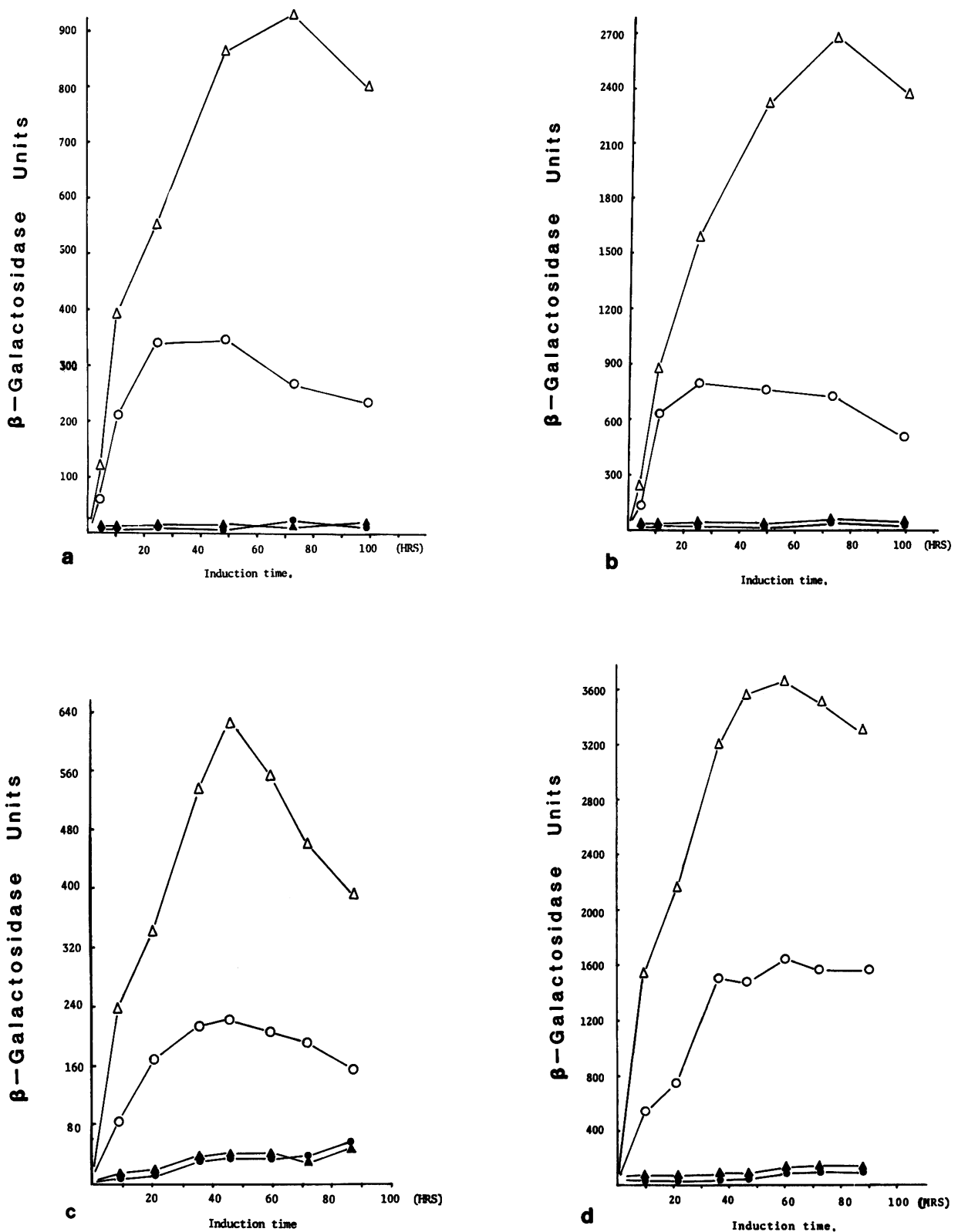
FIG. 8. β-Galactosidase induction of *virB-lacZ* and *virE-lacZ* fusions with (△ and ○) or without (▲ and ●) acetosyringone. (a) *virB-lacZ* fusion pSM243cd contained in A348 (○ and ●) and A281 (△ and ▲); (b) *virE-lacZ* fusion pSM358cd contained in strains A348 (○ and ●) and A281 (△ and ▲); (c) A348 *virB243* MX (○ and ●) and A348 *virB243* MX(pToK9) (△ and ▲); (d) A348 *virE358* MX (○ and ●) and A348 *virE358* MX(pToK9) (△ and ▲). HRS, Hours. MX, Marker exchanged.
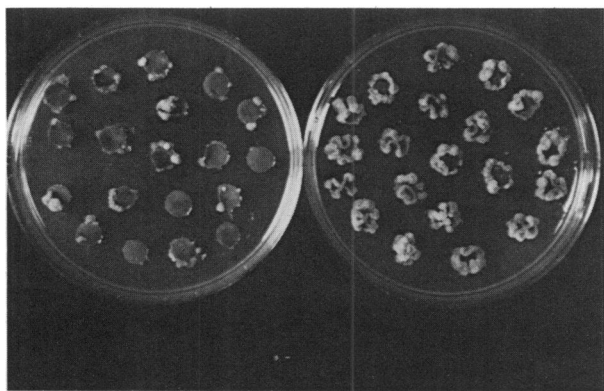
FIG. 9. Comparison of tumor formation of A281(pTVK291) and A281 on *N. glauca* leaf disks. Leaf disks were cocultivated with *A. tumefaciens* for 24 h, and pictures were taken 10 days after inoculation.

diction was confirmed by dot blot analysis in which the same amounts of RNA samples from A348 and A281 after 16 h of cocultivation with plant cells were hybridized with a ^{32}P-labeled pSW163 DNA probe (Fig. 7). Clearly, *virG* was expressed at a much higher level in A281 than in A348, suggesting that the *virG* locus of A281 may have a stronger promoter.

Higher virulence correlates with an increased expression of *vir* genes. For the study of *vir* gene expression in A348 and A281, *virB-lacZ* and *virE-lacZ* fusion plasmids, pSM243cd and pSM358cd, respectively, were mobilized into the two strains. These strains were then induced by acetosyringone. The β-galactosidase levels of both fusions were significantly higher in the A281 background than in the background of A348 (Fig. 8a and b). Since *virG*, a transcriptional activator of these genes, is expressed to a higher level in A281 than in A348, it is likely that the higher β-galactosidase levels of *virB* and *virE* fusions in the A281 background are due to high levels of *virG* expression in A281. Similar data were also obtained for *virD* (data not shown). Indeed, when pToK9 was introduced into A348 *virB243*::Tn*3*HoHo1 and A348 *virE358*::Tn*3*HoHo1, β-galactosidase levels were increased in the resulting strains (Fig. 8c and d).

Since pToK9 conferred a supervirulence phenotype on A348, we conclude that this enhanced virulence correlates with the higher expression of *vir* genes, which most likely results from higher *virG* expression.

Effects of clones of pTiBo542 on A281. The ability of pToK9 to enhance the virulence of A348 raised the question of whether it might be possible to further increase the virulence of A281. As one approach, pToK9 was mobilized into A281, and the virulence of the resulting strain was compared with that of A281 on *N. glauca* leaf disks. The virulence was not enhanced, suggesting that the virulence of A281 could not be increased simply by increasing the copy number of *virG* and the 3' end of *virB*. Therefore, additional fragments of the *vir* region were introduced into A281. These included pTVK291, pTVK255, pTVK85, pTVK178, pTVK320, and pTVK217. When the resulting strains were assayed for their virulence, pTVK291, pTVK85, and pTVK255 increased the virulence of A281 by inducing tumor formation much earlier than in A281 alone (Fig. 9). Furthermore, when *N. glauca* leaf disks were cocultivated for 2 h with A281, A281(pTVK291), A281(pTVK85), A281(pTVK178), A281(pTVK217), A281(pTVK320), A281(pTVK255), A281(pToK9), A281(pVK257), A348(pVK257), and

A348(pTVK291) (Table 3), only A281(pTVK291), A281(pTVK255), and A281(pTVK85) incited tumors. This result indicates that the virulence of A281 can be further enhanced by the addition of the entire *virB* and *virG* operons. This enhancement might involve both an earlier transformation of plant cells and a high efficiency of transformation.

pToK47 enhancement of the virulence of *A. tumefaciens*. Plasmid pToK47 was constructed by subcloning the 15.8-kb *Kpn*I fragment of pTiBo542 into pUCD2, which has a pSa origin and is compatible with P group and Ti plasmids. This plasmid carries the entire *virB*, *virG*, and *virC* operons. As predicted, when pToK47 was mobilized into strains A348, A281, A208, T37, and R1002 (Table 1), all of the resulting strains displayed increased tumor formation on *N. glauca* leaf disks compared with the corresponding strains without the plasmid.

DISCUSSION

Strain A281 was originally characterized as a strain inciting larger tumors than other strains (11). A large tumor could result from (i) faster tumor growth, reflecting stronger oncogenes in T-DNA; (ii) a larger number of initially transformed cells, indicating a higher transformation efficiency; or (iii) rapid transformation, possibly reflecting earlier expression of *vir* genes. Since the growth rates of tumors induced by A281 and A348 are similar once they are formed and since T-DNA clones from A281 did not have any effect on tumor formation by A348, the first possibility is unlikely. In support of this contention, a recent paper (10) demonstrated that a non-T-DNA portion of the Ti plasmid is involved in the supervirulence phenotype of A281. The second possibility is supported by a report that pTiBo542, when used as a source of *vir* genes, gave a higher frequency of transformation in the binary vector system than did other Ti plasmids (2) and by the results of the present study, in which A281 incited tumors all over the edges of *N. glauca* leaf disks, whereas A348 induced tumors only in isolated points on the edges. Evidence favoring the third possibility is the observation that tumors were induced only by A281(pTVK291), A281 (pTVK85), and A281(pTVK255) but not by A281 or A348 following 2 h of cocultivation.

N. glauca leaf disks are very convenient materials on which to compare the virulence of A281 and A348. Since *N. glauca* is a permissive host for *A. tumefaciens* and is susceptible to most *A. tumefaciens* strains (29), *N. glauca* leaf disks can be widely used to assay the level of virulence of different *A. tumefaciens* strains. Since the supervirulence determinant of pTiBo542 was identified primarily by using *N. glauca* leaf disks, we cannot eliminate the possible important roles played by other factors in the case of large tumors or high frequencies of transformation on other plants. However, the supervirulence-conferring fragment contributed to the high virulence on both *N. glauca* and *G. max* cv. Beeson, the latter not being infectible by A348.

Various factors control the host range of *A. tumefaciens* strains. In the limited-host-range strain Ag162, which infects mainly certain varieties of grapevine, the cytokinin biosynthetic gene within the T-DNA and *virA* and *virC* of the *vir* genes are involved in the limitation of host range (31). In our present studies, we found that the 2.5-kb fragment, which increased the efficiency of transformation of A348 on *N. glauca* leaf disks, also extended the host range of A348 to *G. max* cv. Beeson. This result suggests that increasing the expression of certain *vir* genes, at least in certain strains, may increase their virulence and host range.

Since the *virG* locus alone from pTiBo542 could increase the virulence of A348 to a limited extent, whereas the 3' end of *virB* alone had no effect, it is likely that the supervirulence-conferring ability is mainly contributed by high expression of *virG*. However, the supervirulence phenotype required both a high expression of *virG* and the 3' end of *virB*. The function of the 3' end of *virB* is not clear.

Induction assays showed that *virB*, *virD*, and *virE* are expressed to higher levels in the A281 background than in the A348 background. Furthermore, pToK9 could increase the expression of *virB*, *virD*, and *virE* genes in A348. We conclude, therefore, that higher virulence correlates with an increased expression of the *vir* genes. This higher level of *vir* gene expression could be achieved by introducing the transcriptional activator *virG* locus of the supervirulence region. The difference in *virG* expression levels appears to be the primary difference between A281 and A348. Once *vir* genes are expressed at a maximal level, any further increase of *virG* activation will not affect *vir* gene expression, and virulence will not increase. This seems to be the case for strain A281, in which increasing the copy number of *virG* and the 3' end of *virB* alone had no effect. However, the entire *virB* operon and *virG* locus increased the virulence of A281. This finding suggests that the *virB* operon limits the further increase of virulence of A281.

The high efficiency of transformation and the wide host range of *A. tumefaciens* strains are important considerations in applying this system to the genetic engineering of higher plants. Since strain A281 has a higher efficiency of transformation and a broader host range than the usual wide-host-range strains, such as A6 and T37, this strain may prove useful in transforming certain plants that are refractory to infection by the usually employed strains. Furthermore, the merodiploid strain A281 carrying pTVK291, pTVK85, or pTVK255 has an even higher virulence than A281 and may be useful in transforming plants that are refractory even to A281. As our knowledge of the function and regulation of *vir* genes increases, it may be possible to construct strains of *Agrobacterium* spp. that will be capable of transforming plants that thus far have proven to be highly resistant to infection by *Agrobacterium* spp.

ACKNOWLEDGMENTS

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