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Virulence Properties of Strains of *Agrobacterium* on the Apical and Basal Surfaces of Carrot Root Discs

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ABSTRACT

Most pathogenic strains of *Agrobacterium* are able to induce crown gall or hairy root on both the apical surface (facing the root tip) and the basal surface (facing the shoot) of carrot (*Daucus carota* L.) root discs. Tumorigenic strains carrying mutations in the shoot inhibition region of the T-DNA (TL-DNA genes 1 and 2) are markedly attenuated on the basal surface but remain virulent on the apical surface. Coinoculation of two attenuated tumorigenic strains, with mutations in gene 1 and gene 2, respectively, resulted in restoration of virulence on the basal surface. Wild type hairy root-inducing strains can be divided into two groups: those that are virulent on both apical and basal surfaces and those that are virulent only on the apical surface. α-Naphthalene acetic acid stimulated virulence of hairy root strain TR7, belonging to the latter group, on the basal surface. Attenuated virulence on the basal surface can be explained in terms of an auxin deficiency in the basal tissues and unidirectional auxin transport to the apical surface.

Crown gall and hairy root diseases are caused by the soil bacteria *Agrobacterium tumefaciens*, *A. rhizogenes*, and *A. rubi* and affect many dicotyledonous plants. Tumor-inducing (Ti[1]) and root-inducing (Ri) bacterial plasmids are the infectious agents (30, 31). The nomenclature of Holmes and Roberts (10) is used in this paper. In this system, biotypes 1, 2, and 3 of Keane *et al.* (14) and Kerr and Panagopoulos (15) are given the specific epithets *A. tumefaciens*, *A. rhizogenes*, and *A. rubi*, respectively, regardless of whether the plasmids cause crown gall or hairy root. To distinguish between the two diseases, strains are referred to as containing Ti or Ri plasmids.

Wounding of the plant tissues is required for transformation by the bacteria. During plant cell transformation, part of the plasmid, the T-DNA, is inserted into plant nuclear DNA (4, 5). Expression of this T-DNA results in tumor or hairy root formation and also in the synthesis of opines, compounds found specifically in transformed tissue. Several genes in the TL-DNA (the left component of octopine T-DNA) which are common to both octopine and nopaline T-DNA code for tumor morphology functions (3). TL-DNA genes 1 and 2 have been implicated in the control of the auxin balance of transformed tissue (1, 3, 22). The normal function of these genes has been termed shoot inhibition, as mutations in these genes cause shoot formation on tobacco tumors (17). A cytokinin-like function has been ascribed to an adjacent root inhibition gene which, when mutated, gives rise to root formation (16, 22).

The object of the work was to study the crown gall and hairy root transformation process. Differences in virulence properties between wild type strains and strains with mutations in the T-DNA tumor morphology region led to the identification of T-DNA functions differentially affecting virulence on the apical and basal surfaces of carrot root discs. Differences were found among wild type hairy root strains in their virulence properties on carrot root discs. The basis of these differences is not known, but they may be due to functions similar to those of the crown gall T-DNA shoot inhibition region.

MATERIALS AND METHODS

Plant Material. Carrots and parsnips were either purchased locally or specific varieties ('Western Queen,' 'Western Red,' and 'Yates Topweight 556' carrots) obtained from a market garden. The carrots or parsnips were washed, peeled, dipped in ethanol, and flamed. The basal 2 cm of the root were discarded, and discs 5 to 10 mm thick were cut by transverse section, proceeding toward the apex of the root. The outer edges of each disc were trimmed with a scalpel. Discs were placed in sterile specimen containers or Linbro tissue culture trays (12 wells per tray) which contained 10 or 2 ml of 1 or 2% (w/v) agar, respectively, to prevent drying. Care was taken to ensure that discs were correctly placed with either the apical or basal surface facing up, as required. Plant growth substances or antibiotics were added to the agar where specified.

Inoculation. Bacteria were grown for 2 d at 28°C on yeast-mannitol agar. Suspensions of approximately 2×10^9 bacteria/ml were made in sterile distilled H_2O. A small volume (10–50 μl) of this suspension was used to inoculate the cambial area of the apical or basal surface of the carrot or parsnip disc. The inoculated surface faced up except where otherwise indicated. Variation in response between discs taken from the same carrot was small, but variation between individual carrots was large. For this reason, six carrots or parsnips were used as replicates in the survey of wild type strains. Discs were incubated at 25°C under fluorescent light (4.2 μE m^{-2} s^{-1}) and observed regularly between 2 to 6 weeks after inoculation.

The effect of auxin (5×10^{-6} M NAA) on the virulence of hairy root strain TR7 on the basal surface was tested using 50 replicate carrots. Five discs were cut from each carrot, and were used for the following five treatments: (a) uninoculated basal control, (b) uninoculated basal control + NAA, (c) TR7 basal inoculation, (d) TR7 basal inoculation + NAA, (e) TR7 apical inoculation (positive control). The inoculated surface faced downward on water agar or water agar containing 5×10^{-6} M NAA. Root formation was assessed 56 d after inoculation. In some cases, roots which developed were tested for the presence of opines by HVPE of ethanolic extracts, as described below under "Detection of Opines."

[1] Abbreviations: Ti, tumor-inducing; Ri, root-inducing; pTi, tumor-inducing plasmid; pRi, root-inducing plasmid; NAA, α-naphthalene acetic acid.

A quantitative comparison of the virulence of strains with T-DNA mutations was made using the following method. Fourteen discs, 5 mm thick, were cut from each of eight carrots (eight replicates). The discs from a single carrot were randomly assigned to seven treatments (six strains and one water control) which were each applied to the apical and to the basal surface (total 7 × 2 = 14 discs). The discs were inoculated 24 h after cutting, and tumor growth was measured 4 weeks after inoculation by (a) photography, to enable the proportion of the carrot disc cambium covered by tumor growth to be measured, and (b) removing the tumor tissues with a scalpel and weighing. The experiment was carried out twice: first with carrots of smaller diameter using Linbro 12-well tissue culture trays (well diameter, 24 mm) for incubation of the discs, and second with carrots of larger diameter, incubating the discs in sterile specimen containers (diameter, 43 mm) of 70-ml capacity.

Axenic Plant Tissue. In order to obtain tumor tissue free from bacteria, tumor slices 1 to 2 mm thick were placed on Monnier's medium (20) with Morel's vitamins (21) in 0.8% (w/v) agar containing carbenicillin (1 mg/ml) for 4 weeks. Tissues free of bacteria were maintained on medium without carbenicillin. Tissue growth was measured nondestructively by area determination from a photograph.

Detection of Opines. Opines were extracted by homogenizing plant tissue with ethanol (1 ml/g fresh weight). After centrifugation, approximately 10 μl of the supernatant solution was separated by paper electrophoresis on Whatman No. 1 paper using the apparatus of Tate (27), employing the following buffers: 0.75 M HCOOH/1 M acetic acid (pH 1.7) and 0.2 M NH_4HCO_3/0.1 M NH_4OH (pH 9.2). Mobilities are expressed relative to Orange G (1.0) with fructose as the nonmigrating marker. Agropine, N^2-(1′-deoxymannityl)-L-glutamate (dManlGlu) and N^2-(1′-deoxymannityl)-L-glutamine (dManlGln) (23) were detected using the alkaline silver nitrate dip reagent of Trevelyan *et al.* (29).

Bacterial Strains. Strains carrying T-DNA mutations were provided by Dr. D. Garfinkel, University of Washington, Seattle, and Prof. J. Schell, Max Planck Institut für Züchtungsforschung, Cologne. Sources of wild type strains are included in Table I.

Table I. *Virulence Properties of Agrobacterium Strains*

Wild type *Agrobacterium* strains are classified according to basal virulence properties on carrot root discs. Bas$^+$ strains are virulent on both apical and basal surfaces; Bas^{att} strains are attenuated on the basal surface (facing the shoot) but virulent on the apical surface (facing the root tip). Sources of strains are listed below. Strains *A. tumefaciens* IIBNV6 and *A. rhizogenes* TR107 were avirulent on both apical and basal surfaces.

	Bas$^+$	Bas^{att}
A. tumefaciens (= biotype 1)	pTi A6, B6, C58, Ach5, IIBV7, TR108, 15955, T37, 4, 925, 1641	pTi TR104 pRi 2655, 2657, 2659
A. rhizogenes (= biotype 2)	pTi 27, 29, 35, 49, TT133, 108 pRi TR105, A4, 15834	pRi TR7, 8196, TR101, 11325
A. rubi (= biotype 3)	pTi Ag84, 305, 308, 374	

Sources of strains: 15955 and 11325, American Type Culture Collection, Rockville, MD; TR101 and TR107, J. DeLey, Ghent, Belgium; C58, R. Hamilton, PA; TR104, TR108, TT133, TR105, and TR7, International Collection of Phytopathogenic Bacteria, Davis, CA; 27, 29, 35, 49, 108, 305, 308, and 374, A. Kerr, Adelaide, Australia; 8196, 15834, and IIBNV6, J. Lippincott, Evanston, IL; A6, B6, IIBV7, and T37, G. Morel, Versailles, France; A4, L. Moore, Corvallis, OR; 4, 925, 1641, 2655, 2657, and 2659, National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.; Ag84, C. Panagopoulos, Athens, Greece; Ach 5, J. Schell, Cologne, W. Germany.

RESULTS

Inoculation with Wild Type Strains. Table I shows the classification of wild type *Agrobacterium* strains according to their virulence properties on the basal surface of carrot root discs. Most pathogenic strains were able to induce tumors or hairy root on both the apical surface (facing the root tip) and the basal surface (facing the shoot). These strains are referred to as Bas$^+$ (Fig. 1A). Some wild type strains, however, were either avirulent or much attenuated on the basal surface; we call these strains Bas^{att} (Fig. 1B). Of 22 wild type pTi strains drawn from all three species of *Agrobacterium*, only one (*A. tumefaciens* TR104, a slow-growing octopine strain) was Bas^{att}. In contrast, pRi strains were more commonly Bas^{att} than Bas$^+$. The Bas^{att} phenotype was observed irrespective of whether the inoculated surface faced up or down on the agar.

On uninoculated carrot discs a callus usually formed around the cambium, but only on the apical surface (Fig. 1C). In the case of pRi strains, root formation was used as a morphological marker to demonstrate that transformation of the apical surface had indeed occurred. For pTi strain TR104, apical transformed tissue was distinguished from untransformed callus tissue by hormone-independent growth on culture medium and by the presence of silver nitrate-positive opines, which were detected by paper electrophoresis of ethanol extracts at pH 1.7 and pH 9.2.

Callus growth was observed on the phloem of the basal surface of uninoculated discs where a lateral root was close to the basal cut surface. Tumors or hairy roots were also able to develop in such situations on discs inoculated with Bas^{att} strains.

Parsnip root discs responded in the same way as carrot discs to inoculation with Bas$^+$ and Bas^{att} strains. No difference in response was found between carrot varieties tested.

T-DNA Tumor Morphology Mutants of pTi Strains. Tables II and III show the virulence on carrot root discs of octopine strains of *Agrobacterium* carrying different TL-DNA mutations. Figure 2 shows the location of mutations on the TL-DNA of the octopine Ti plasmid. Virulence was assessed by measuring (a) tumor weight per disc and (b) the proportion of cambial circumference covered by tumors. There was good agreement between results obtained by these two different methods. Tumor weights were higher in experiment 2 than in experiment 1 due to the larger diameter of the carrots used. In each experiment there was large variation between carrots with respect to the amount of tumor tissue formed.

Wild type parent octopine strains A6 and B6 were virulent on both the apical and basal surfaces (Bas$^+$). Strains with mutations tmr-338 and tmr-149 in the root inhibition region (TL-DNA gene 4) were also Bas$^+$.

Strains with shoot inhibition mutations in either gene 1 or gene 2, or both, (insertion mutants tms-328, tms-394, and A66, and deletion mutants pGV2206, pGV2216, and pGV2219) were markedly attenuated or avirulent on the basal surface (Bas^{att}) but were virulent on the apical surface. Virulence of the Bas^{att} mutant strains was decreased to a greater extent on the basal surface than on the apical surface, relative to the virulence of the wild type parents. This was more clearly seen when using the data for the proportion of cambium covered by tumors than when tumor weight was used as a measure of virulence. An exception was in experiment 1 with mutant strain pGV2216 where low virulence was recorded on both surfaces by both methods. Tumor weight measurements do not take into account variation in the size of

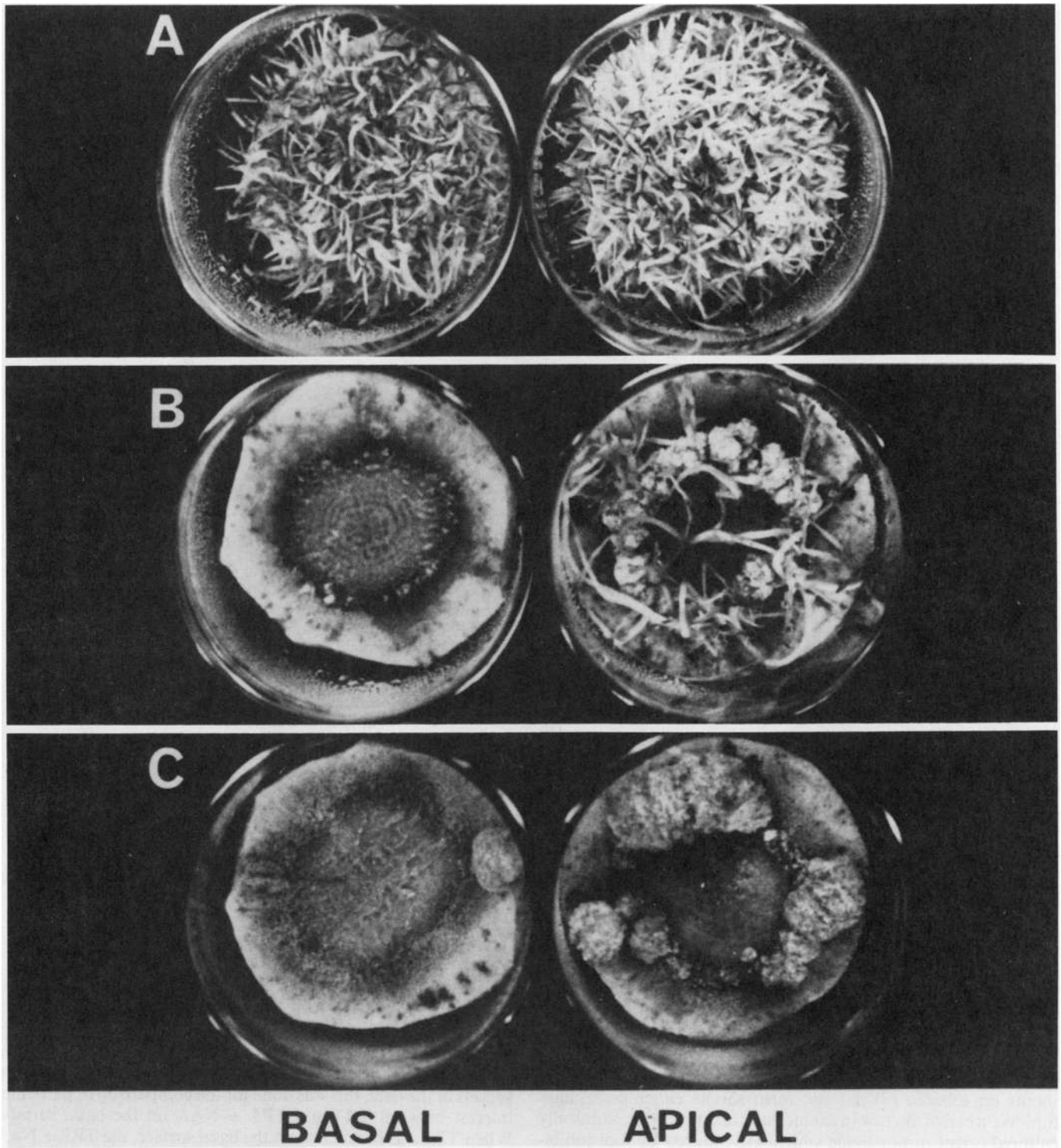

FIG. 1. Inoculation of hairy root on parsnip discs, photographed 40 d after inoculation. A, TR105 is highly virulent on both apical and basal surfaces; B, TR7 is avirulent on the basal surface and mildly virulent on the apical surface; C, uninoculated discs showing callus formation on the apical cambium but not on the basal cambium. Callus has also developed on a lateral root near the cut surface of the phloem of the basal surface.

discs and the associated difference in the amount of cambium available for tumor formation. The virulence of Bas^{att} strains on the apical surface was confirmed by the detection of opines of the agropine family in ethanol extracts of apical tissues. The agropine family of opines includes agropine, dManlGlu, and dManlGln, which can be used as chemical markers for transformed tissue incited by octopine strains (24).

The virulence of the strain carrying the deletion pGV2210, in which genes 1 and 4 are inactive (17), was low on the apical surface but opines of the agropine family were detected, indicating that transformation must have occurred.

A strain carrying the double deletion pGV2224, which combines the deletions of pGV2210 and pGV2219 and is therefore mutated in genes 1, 2, and 4, exhibited even lower virulence on the apical surface. Ethanol extracts of apical tissues from discs inoculated with pGV2224 contained no detectable agropine, dManlGlu, dManlGln, or octopine.

Axenic carrot tumor tissue transformed by gene 1 mutant tms-328 grew rapidly when first placed on culture medium, but growth had almost ceased after 6 months in culture. Growth could be restored by addition of auxin (10^{-7} M 2, 4-D) as shown by an experiment in which uniform pieces of tms-328 axenic carrot tissue were placed on Monnier's medium and on Monnier's medium + 10^{-7} M 2,4-D. After 20 d, the mean areas per

Table II. *Virulence, on Carrot Root Discs, of Strains of Agrobacterium Carrying TL-DNA Deletion Mutations*

Bacteria were inoculated on the apical and basal surfaces of carrot root discs and the tumors were harvested and weighed 4 weeks after inoculation. The proportion of cambial circumference covered with tumors was measured from a photograph taken immediately before tumors were harvested. The wild type parent strain (positive control) harbored the octopine B6 Ti plasmid pGV3111, and the deletion mutants were derived from this plasmid (17). The negative H_2O control was uninoculated. Experiment 1, mean of seven replicates; experiment 2, mean of eight replicates.

Ti Plasmid or Mutant Ti Plasmid	Mean Tumor Wt				Cambial Circumference Covered with Tumors			
	Exp. 1		Exp. 2		Exp. 1		Exp. 2	
	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
	mg/disc				%			
pGV3111	86	61	407	252	69	34	86	68
pGV2206	56	0.7	227	6	46	2	65	3
pGV2216	14	0.0	84	0.3	14	0	40	0
pGV2219	56	0.3	193	1.5	45	0	58	4
pGV2210	9	0.0	44	0.2	18	0	25	0
pGV2224	6	0.0	11	0.0	2	0	11	0
H_2O control	15	0.0	26	0.0				

Table III. *Virulence, on Carrot Root Discs, of Strains of Agrobacterium Carrying TL-DNA Insertion Mutations*

Bacteria were inoculated on the apical and basal surfaces of carrot root discs and the tumors were harvested and weighed 4 weeks after inoculation. The proportion of cambial circumference covered with tumors was measured from a photograph. A strain harboring the A6 Ti plasmid was the positive control and the insertion mutants were derived from this plasmid (2, 7). All plasmids were in the same genetic background (strain C58C1). The negative H_2O control was uninoculated. Experiment 1, mean of eight replicates; experiment 2, mean of five replicates.

Ti Plasmid or Mutant Ti Plasmid	Mean Tumor Wt				Cambial Circumference Covered with Tumors			
	Exp. 1		Exp. 2		Exp. 1		Exp. 2	
	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
	mg/disc				%			
A6	67	22	346	177	43	34	78	66
A66	23	0.0	191	5	28	0	36	0
tms-394	47	0.0	315	21	45	0	38	7
tms-328	27	0.7	157	10	52	1	65	5
tmr-149	42	38	259	219	71	41	87	70
tmr-338	71	48	265	179	81	52	84	75
H_2O control	21	1.0	36	0				

tissue piece were (a) Monnier's − 2,4-D: 0.52 ± 0.12 cm^2 (13 pieces) and (b) Monnier's + 2,4-D: 1.46 ± 0.48 cm^2 (11 pieces). On carrots, this mutant tumor tissue, which is known to form shoots on tobacco (7) did not form shoots either as primary tumors on carrot discs or in axenic culture. In contrast, axenically cultured carrot tumor tissue which was induced by root inhibition mutants tmr-338 and tmr-149 developed roots which were able to grow without addition of plant growth substances.

Effect of Auxin on Basal Virulence of Hairy Root Strain TR7 on the Basal Surface. In order to test the effect of auxin on the virulence of TR7 (Bas^{att}) on the basal surface, NAA (pH6) was supplied at 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M in agar to carrot discs inoculated on the basal surface with TR7 bacteria. The inoculated surface faced the agar. Control inoculations of TR7 on the apical surface showed good root growth with the inoculated surface facing the agar. When the basal surface was inoculated with TR7, roots formed when NAA was present at 10^{-6} and 10^{-5} M. These roots contained dManlGlu and dManlGln, the opines characteristic of roots induced by strain TR7 (24).

In a subsequent experiment, 5×10^{-6} M NAA was used and the results are shown in Table IV. A χ^2 test revealed highly significant differences ($\chi^2 = 90.5$, $P < 0.001$) between the five treatments. Although it is not usual to perform repeated tests on subsets of the data, this was done for the comparison of particular interest between TR7 and TR7 + NAA on the basal surface. When TR7 was inoculated on the basal surface, the TR7 + NAA treatment induced the formation of roots on more discs than TR7 alone and the results for the two treatments were significantly different ($\chi^2 = 10.3$, $P < 0.01$). Auxin stimulated hairy root growth on 36% (=18/50) of carrots but the amount of root growth was less than on control apical inoculations in almost all cases. The presence of marker compounds dManlGlu and dManlGln (M_{OG} − 0.28 at pH 1.7) in the roots induced on the basal surface indicated that they were transformed roots.

Complementation between Bas^{att} T-DNA Mutants. Two Bas^{att} strains with the TL-DNA mutations tms-394 (a gene 2 mutant) and tms-328 (a gene 1 mutant) were inoculated on the basal surface of carrot root discs at the same time and in approximately equal bacterial cell densities. This coinoculation resulted in the formation of more primary tumors, covering a larger area of the

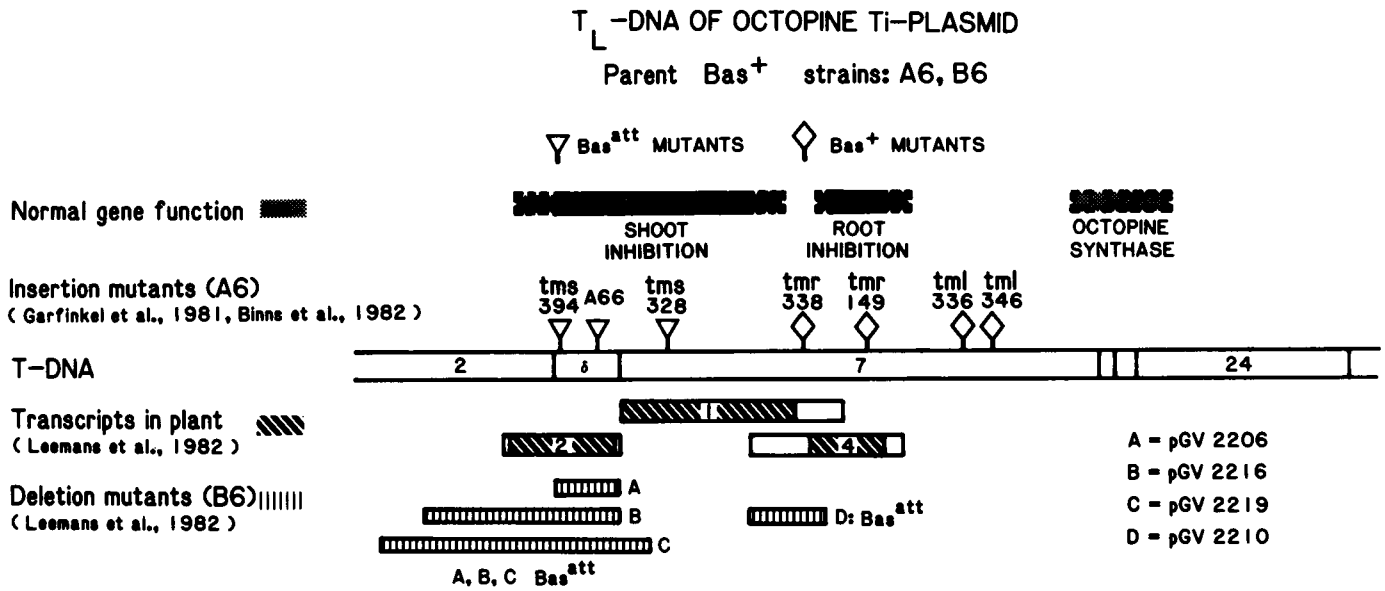

FIG. 2. Virulence properties of T-DNA tumor morphology mutants of pTi strains A6 and B6 of *Agrobacterium*. The TL-DNA of the octopine parent strains is presented as the Eco RI restriction map of Thomashow *et al.* (28). The positions of the 'normal gene function' regions are taken from Garfinkel *et al.* (7). Mutations causing attenuation of virulence on the basal surface (Bas^{att}) map in the shoot inhibition region. TL-DNA mutations referred to as Bas^{+} do not decrease virulence on the basal surface. The map positions of deletion mutations are from Leemans *et al.* (17), and the designations used in this figure are: A = pGV2206; B = pGV2216; C = pGV2219; D = pGV2210.

Table IV. *Effect of Auxin on Hairy Root Induction on Carrot Discs Inoculated with Strain TR7*

Fifty carrots were used as replicates and five discs from each used for the five treatments. The inoculated surface faced water agar or water agar containing 5×10^{-6} M NAA. Root formation was assessed 56 days after inoculation.

Treatment	Inoculated Surface	No. of Discs with Roots on Inoculated Surface	Total Discs
Water	Basal	0	50
Water + NAA	Basal	0	50
TR7	Basal	5	50
TR7 + NAA	Basal	23	50
TR7	Apical	41	50

cambium, than when either mutant strain was inoculated by itself. The amount of cambium (percentage of circumference) covered by tumor growth was measured 40 d after inoculation. Results for the three treatments were as follows (mean of four carrots): (a) tms-328: 2.7%, (b) tms-328 + tms-394: 47.5%, (c) tms-394: 2.7%. These two strains, with mutations in different genes of the shoot inhibition region, were thus able to complement each other and restore virulence on the basal surface. The results were statistically significant ($H = 6.49$, $P < 0.05$) as determined by the Kruskal-Wallis one-way analysis of variance by ranks.

DISCUSSION

Natural callus formation on the apical surface of uninoculated carrot discs (8) is a result of cell division in the cambium and, in its unorganized shape, the callus resembles crown gall tumor tissue. This callus does not develop on the basal surface, indicating an intrinsic polarity in the carrot disc which is unaffected by the orientation of the disc.

Klein and Tenenbaum (16) noted that the amount of crown gall tumor tissue formed on the apical surface (facing the root tip) was twice that formed on the basal surface, whereas DeRopp (6) reported no difference between the two surfaces in the amount of tumor tissue formed. Magnus (19) observed either the former or the latter type of result, depending on the carrot variety used. The botanical term polarity was used in this early work to describe differences in amount of tumor tissue on the two surfaces. The spectacular and morphologically unequivocal polarity with hairy root strains (Fig. 1) has not been previously reported. We have avoided using the term polarity which has other connotations in the crown gall literature and prefer to use Bas^{+} and Bas^{att} to refer to tumor or hairy root formation on the basal surface, with virulence on the apical surface being implied. Wild type Bas^{att} strains, including one tumor-inducing and several hairy root-inducing strains, are almost avirulent on the basal surface (Fig. 1).

Both callus formation and virulence of Bas^{att} strains appear to be auxin-requiring, and auxin level is the only factor found thus far to increase the virulence of Bas^{att} strains on the basal surface. Addition of auxin (NAA) to inoculations of hairy root strain TR7 on the basal surface stimulated formation of transformed roots although not in all replicates. NAA was effective at concentrations between 10^{-6} and 10^{-5} M. Leemans *et al.* (17) and Joos *et al.* (13) reported restoration of virulence of Bas^{att} T-DNA mutants on carrot discs and potato discs by addition of auxin, although no quantitative data were presented. The T-DNA regions involved in attenuation of basal virulence are genes 1 and 2, which appear to control the auxin balance of tumor tissue (3). A lack of auxin at the basal surface could provide an explanation for the decreased ability of Bas^{att} strains to form tumors on that surface.

It is a widely held view that auxin transport in plants is unidirectional (polar) from shoot tip to root tip (9). Higher levels of auxin at the apical than at the basal surface of carrot root discs have been measured (25). Pilet (25) provided evidence that this was due to increased auxin synthesis at the apical surface and increased catabolism at the basal surface. This is in contrast to the view of Gautheret (8) that unidirectional auxin transport toward the apical surface was responsible for apical callus growth. It is conceivable that both mechanisms operate together to

account for larger amounts of auxin at the apical than the basal surface. The results presented here are further evidence for higher levels of auxin at the apical surface than at the basal surface. There is now clear evidence (12) that the auxin transporter in pea stems is present only at the lower ends of the cells (facing the root tip). Such a situation, if demonstrated in carrot cells, would provide a rational explanation for the Bas^{att} phenotype.

Auxin could conceivably act either before or after transformation in stimulating virulence of Bas^{att} strains. Axenic tissue induced by the strain carrying TL-DNA gene 1 mutation tms-328 is auxin-dependent, and hence auxin must be effective after transformation in this case. Avirulent mutant strains with large T-DNA deletions can transform plant cells without subsequent tumor formation. Evidence for this is the detection of octopine synthase in wound tissue of carrot discs inoculated with an avirulent strain carrying the large TL-DNA double deletion pGV2224 (17). No opines were found in our apical tissue inoculated with pGV2224 but the octopine synthase assay is a more sensitive test for transformation. Our inability to detect agropine in this tissue could be due to loss of TR-DNA, a not infrequent occurrence (28). Transfer of T-DNA to the plant cell by avirulent strains suggests that Bas^{att} strains transform cells at the basal surface and that auxin stimulates their subsequent growth. However, the possibility of a role for auxin before or at transformation is not excluded. The expression of TL-DNA genes by agrobacteria (26) raises the interesting possibility that the bacteria may play an active role in inducing plant cell division as part of the transformation process.

Mutations in TL-DNA genes 1 and 2 decreased the virulence of agrobacteria on carrot root discs to a greater extent on the basal than on the apical surface. Strains mutated in gene 4 (root inhibition) did not show a decreased virulence on either surface but inactivation of both genes 1 and 4, as in the deletion mutant pGV2210, led to a marked decrease in virulence on both the apical and basal surfaces. This suggests that the small TL-DNA transcript situated between genes 1 and 4 and which is expressed only in the bacteria (26) may play a role in virulence on the apical surface.

The wild type Bas^{att} pRi strain TR7 and the Bas^{att} T-DNA shoot inhibition mutants are similar in their response to auxin but the particular property of the Ri plasmid causing the attenuated virulence of TR7 on the basal surface has not been identified. A detailed analysis of the TR7 Ri plasmid is required in order to determine whether or not it contains regions showing homology to the shoot inhibition region of Ti plasmids. Such regions have been found on the Ri plasmid of the Bas^{+} hairy root strain A4 (11). Attenuated virulence on the basal surface could be explained if these shoot inhibition functions are absent from the Ri plasmids of Bas^{att} strains. Chromosomally-coded functions may also play a part in differences in virulence properties between the hairy root strains.

There are basic differences between pRi and pTi strains which are as yet unexplained in molecular terms. For example, Bas^{att} pRi strains are root-inducing, yet the pTi Bas^{att} T-DNA mutants are shoot-inducing on tobacco (2, 7), despite the similar responses of these strains to auxin as mentioned above.

The complementation observed between strains with mutations tms-328 and tms-394 in the shoot inhibition region differs from previously reported complementation (23) between strains with mutations in the T-DNA morphology region in two respects. First, tms-328 and tms-394 are mutations in different genes (genes 1 and 2) of the same (shoot inhibition) region of the T-DNA, and second the complementation reported here resulted in the restoration of virulence in a situation where each mutated strain by itself was markedly attenuated. The complementation observed by Ooms *et al.* (23), on the other hand, involved the formation of tumors with wild type morphology upon coinoculation of tobacco stems with a root inhibition and a shoot inhibition mutant. The complementation observed here between tms-328 and tms-394 was not due to bacterial conjugation and T-DNA recombination (S. C. Donner, unpublished data) and could, therefore, be due to either double infection of a single plant cell or to cross-feeding between transformed cells from separate transformation events as suggested by Ooms *et al.* (23). Alternatively, evidence for production of plant growth substances by agrobacteria due to bacterial expression of TL-DNA genes (26) suggests that complementation between bacterial mutant strains for production of such substances may occur.

The list of wild type Bas^{att} strains is almost identical to the list of 'receptor' strains identified by Lippincott and Lippincott (18) in their mixed inoculation studies on Pinto bean leaves. Their receptor strains were 8196, TR7, TR101, 11325, IIBNV6, and TR104 (=ATCC13333). If the same factor(s) were involved both in differential apical/basal virulence on carrot root discs and in Pinto bean leaf complementation, then strain IIBNV6, a receptor strain on Pinto bean leaves, should be virulent on the apical surface of carrot root discs. In the present study, no opine markers were detected in apical tissues inoculated with IIBNV6, nor were such tissues able to grow in culture in the absence of plant growth substances. Strain IIBNV6 may lack more function(s) necessary for tumorigenic ability than do the Bas^{att} strains listed in Table I.

The auxin-dependence of axenic tissue of tms-328 on carrot is in marked contrast to the results of Joos *et al.* (13), who found no auxin-dependent lines from tumors induced by mutants in genes 1 or 2. These results were apparently obtained using tobacco, where mutants in genes 1 and 2 induce formation of tumors with shoots which would provide a source of auxin. Binns *et al.* (2) reported, also for tobacco, that tumors induced by mutant strain A66 (Bas^{att}) are auxin-dependent in culture unless buds are formed. The reason for the auxin-dependence of tms-328 carrot tissue may lie in its inability to form shoots which would be a potential supply of auxin.

Although the reason for basal hairy root attenuation is not known, the genetic analysis of the crown gall TL-DNA has shown that the basal tumor attenuation phenotype is caused by mutations in genes 1 and 2. The basal tumor attenuation phenotype may well provide a suitable screening test for T-DNA mutations in these genes. T-DNA analysis of A66 (2) revealed an insertion in gene 2, and a similar analysis of the Ti plasmid of TR104 (also Bas^{att}) is warranted.

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