

Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of Development of the Hypersensitive Response Elicited by *Pseudomonas syringae* pv. *phaseolicola*

DAVID ROBINETTE† AND ANN G. MATTHYSSE*

Department of Biology, Coker Hall, CB 3280, University of North Carolina,
Chapel Hill, North Carolina 27599

Received 2 April 1990/Accepted 23 July 1990

Injection into tobacco leaves of biotype 1 *Agrobacterium tumefaciens* or of *Pseudomonas savastanoi* inhibited the development of a visible hypersensitive response to the subsequent injection at the same site of *Pseudomonas syringae* pv. *phaseolicola*. This interference with the hypersensitive response was not seen with injection of bacterial growth medium or *Escherichia coli* cells. Live *A. tumefaciens* cells were required for the inhibitory effect. Various mutants and strains of *A. tumefaciens* were examined to determine the genes involved. Known chromosomal mutations generally had no effect on the ability of *A. tumefaciens* to inhibit the hypersensitive response, except for *chvB* mutants which showed a reduced (but still significant) inhibition of the hypersensitive response. Ti plasmid genes appeared to be required for the inhibition of the hypersensitive response. The bacteria did not need to be virulent in order to inhibit the hypersensitive response. Deletion of the *vir* region from pTi had no effect on the inhibition. However, the *T* region of the Ti plasmid was required for inhibition. Studies of transposon mutants suggested that the *tms* but not *tmr* or *ocs* genes were required. These genes were not acting after transfer to plant cells since they were effective in strains lacking *vir* genes and thus unable to transfer DNA to plant cells. The results suggest that the expression of the *tms* genes in the bacteria may inhibit the development of the hypersensitive response by the plant. An examination of the genes required in *P. savastanoi* for the inhibition of the hypersensitive response suggested that bacterial production of auxin was also required for the inhibition of the hypersensitive response by these bacteria.

The hypersensitive response (HR) in plants results when pathogenic species of bacteria or fungi enter the tissues of a nonhost plant species or a resistant cultivar of a host species. Nonphytopathogenic and saprophytic bacteria or fungi do not generally elicit a hypersensitive response. Current evidence suggests that the plant cells become committed to the development of an HR within the first 2 to 4 h after inoculation of the eliciting microorganisms. The HR is characterized by loss of turgor pressure and electrolyte leakage from plant cells in the inoculated area within the first few hours. Tissue browning, collapse, and necrosis of the cells in this area are generally visible within 18 h after inoculation of the eliciting bacteria. The bacteria are limited to the area originally inoculated and do not spread beyond its edges. The HR is thought to function in the limitation of the growth of the microorganism and to be associated with disease resistance (10).

Although most phytopathogenic bacteria elicit an HR on resistant plants, no HR is generally observed on susceptible plants. Thus, *Agrobacterium tumefaciens* does not elicit an HR on most dicots on which it induces tumors. In addition, biotype I *A. tumefaciens* fails to elicit a typical hypersensitive response on resistant plants.

A. tumefaciens is a soil bacterium which normally invades plants at wound sites and which in host plants produces tumors known as crown galls. In nature, many other microorganisms would also be expected to enter a wound site on a plant. While many of these organisms might be saprophytes, some of them are probably pathogens for other plant

species (incompatible pathogens). The invaded plant would then produce an HR in response to the microorganism. This HR might then interfere with the development of crown gall tumors by *A. tumefaciens*. Although estimates of the time required for the induction of tumors by *A. tumefaciens* vary from 4 to 8 h for *Bryophyllum diageomontiana* (22) and *Petunia hybrida* (23) to more than 30 h for *Vinca rosea* (1), it appears that the HR is generally a faster response than tumor transformation. Thus, if *A. tumefaciens* is able to induce tumors in such mixed infections, it seems possible that *A. tumefaciens* might prevent the host plant from producing an HR which would result in the death of the plant cells surrounding the wound site before they could be transformed to crown gall tumor cells.

This study was undertaken to determine whether *A. tumefaciens* was capable of preventing the HR elicited by an incompatible bacterial pathogen. In addition, the genetic basis for the ability of *A. tumefaciens* to inhibit the HR was examined.

MATERIALS AND METHODS

The bacterial strains used are listed in Table 1. Additionally, a nontoxigenic derivative of *Pseudomonas syringae* pv. *phaseolicola*, strain G50T Tox⁻ obtained from S. Patil, University of Hawaii, was used to elicit an HR in *Nicotiana tabacum* (17), and a transposon Tn5 mutant of *P. syringae* pv. *phaseolicola* which is unable to elicit an HR (Hyr-25 [4]) was used to examine the effect of *P. syringae* pv. *phaseolicola* on tumor formation by *A. tumefaciens*. For experiments involving recovery of the bacteria from leaf tissue, a derivative of G50T Tox⁻ carrying a random Tn5 insertion into the chromosome was used. This transposon insertion had no

* Corresponding author.

† Present address: Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC 27599.

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source or reference
<i>E. coli</i>		
E6	C600(pRK2051)	S. Gelvin
E207	C600(pE207) ^a	S. Gelvin
<i>A. tumefaciens</i>		
A6	Wild type	A. Braun
C58	Wild type	E. Nester
ACH5	Wild type	J. Schell
15955	Wild type	American Type Culture Collection
B6	Wild type	A. Braun
1D1	Wild type	C. Kado
ACH5C3	ACH5 cured of pTi, avirulent	J. Schell
NT1	C58 cured of pTi, avirulent	E. Nester
NT1(pE207)	NT1 carrying pE207 (pTi <i>EcoRI</i> fragment 7 cloned in pRK2501)	S. Gelvin
Cel-1	A6 with Tn5, cellulose-minus, virulent	11
Cel-12	A6 with Tn5, cellulose-minus, virulent	11
Att-C43	C58 with Tn5, attachment-minus, avirulent	12
Att-C69	C58 with Tn5, attachment-minus, avirulent	12
A1038	C58 with Tn5, <i>chvB</i> , avirulent	5
A1045	C58 with Tn5, <i>chvB</i> , avirulent	5
A2507	C58 with Tn5, <i>chvB</i> , avirulent	5
At91	C58 with Tn5 in <i>ocs</i> , virulent	S. Gelvin (6)
At121	C58 with Tn3 in <i>tms-2</i> , virulent	S. Gelvin (6)
At122	C58 with Tn5 in <i>tms-1</i> , virulent	S. Gelvin (6)
At123	C58 with Tn5 in <i>tmr</i> , virulent	S. Gelvin (6)
At37	LBA4404(pAL4044), plasmid carries <i>vir</i> region of pTi, <i>T</i> region deleted	S. Gelvin (8)
At38	LBA1050(pAL1050), plasmid carries <i>T</i> region of pTi, <i>vir</i> region deleted	S. Gelvin (8)
SATC22	1D1(pSa), avirulent, auxin reduced	C. Ireland-Valentine
<i>Rhizobium meliloti</i>		
1027	Wild type	S. Long
1322	Wild type with Tn5	F. Ausubel
<i>P. savastanoi</i>		
PB213	Wild type	T. Kosuge (21)
PB213-16	Cytokinin-minus	T. Kosuge (21)
PB213-3	Auxin-minus, cytokinin-reduced	T. Kosuge (21)

^a Plasmid pE207 is pRK2501 carrying pTi *EcoRI* fragment 7.

effect on bacterial growth rate, virulence, or ability to induce the HR.

P. syringae pv. phaseolicola and *A. tumefaciens* cultures were grown at 25°C in Luria broth containing (per liter) 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 0.3 ml of 3 M NaOH (11). *Pseudomonas savastanoi* strains were grown at 25°C in nutrient broth (Difco) containing 5% sucrose (21). *N. tabacum* cultivar Coker 319, *Bryophyllum diagremontiana*, and *Phaseolus vulgaris* UI34 were grown in the greenhouse.

In order to test the effect of *P. syringae* pv. phaseolicola on tumor formation by *A. tumefaciens*, approximately 10⁹ bacteria grown for 2 days on Luria agar were inoculated into toothpick wounds on leaves of *B. diagremontiana*. For sequential inoculations, the time between inoculations was 4 h. Inoculated plants were observed for the development of tumors for the next 8 weeks.

Bacterial cultures to be tested for their ability to inhibit the development of the HR were injected with a hypodermic syringe into four sites on each of three leaves of a tobacco plant. The tobacco plants used were between 8 and 20 weeks old and 40 to 150 cm tall. The leaves used were between 10 and 18 leaves from the top of the plant and 15 to 30 cm long. The primary leaves of bean plants were injected when they were between 1 and 2 cm long. The injected sites were labeled with a nontoxic colored marker. As a control, sterile bacterial culture medium was injected into four sites on the same leaves of tobacco and one site on bean leaves and the sites were labeled. The plant was left undisturbed for 4 h

after the initial injections. At this time, 10 to 100 µl of nontoxic *P. syringae* pv. phaseolicola G50T at concentrations of 1 × 10⁹, 5 × 10⁸, and 2 × 10⁸ bacteria per ml were injected into each leaf within the sites previously injected with the test bacteria or with culture medium. As an additional control, each concentration of G50T was injected into sites on each tobacco leaf where no previous injections had been made. The G50T injection sites were labeled, and the

TABLE 2. Effect of *P. syringae* pv. phaseolicola on tumor formation by *A. tumefaciens*

Bacterial strain(s) inoculated ^a		% of inoculated sites developing tumors ^b
First inoculum	Second inoculum	
<i>P. syringae</i> pv. phaseolicola G50	None	0 ^c
<i>A. tumefaciens</i> C58	None	100
<i>A. tumefaciens</i> C58	<i>P. syringae</i> pv. phaseolicola G50	88
<i>P. syringae</i> pv. phaseolicola G50	<i>A. tumefaciens</i> C58	6 ^c
<i>P. syringae</i> pv. phaseolicola Hyr-25	<i>A. tumefaciens</i> C58	63

^a Inoculations into *B. diagremontiana* leaves were made as described in Materials and Methods.

^b Percentage of inoculated sites developing tumors within 8 weeks. More than 15 sites were scored for each treatment.

^c A visible HR (tissue browning and collapse) was seen at the inoculated sites.

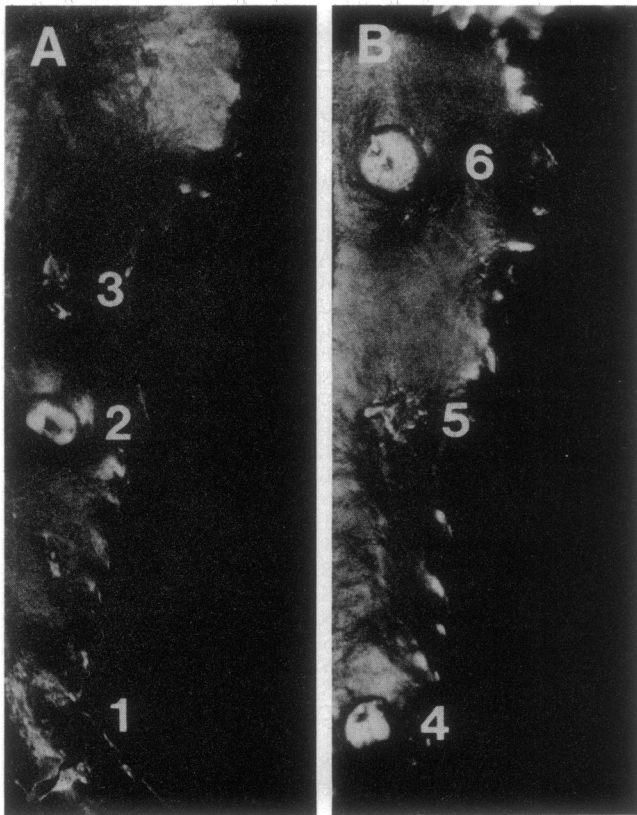


FIG. 1. Effect of *P. syringae* pv. phaseolicola on tumor formation by *A. tumefaciens* C58 on leaves of *B. diageomontiana*. The leaves were photographed 5 weeks after they were inoculated. Sites: 1, *P. syringae* pv. phaseolicola G50 alone; 2, *A. tumefaciens* C58 alone; 3, *P. syringae* pv. phaseolicola G50 first, *A. tumefaciens* second; 4, *P. syringae* pv. phaseolicola Hyr-25 first, *A. tumefaciens* second; 5, *P. syringae* pv. phaseolicola G50 and *A. tumefaciens* together; 6, *A. tumefaciens* first, *P. syringae* pv. phaseolicola second. Tissue collapse and an HR were visible at sites 1 and 3 after 48 h. The delay between first and second inoculations was 4 h. Note that the prior inoculation of *P. syringae* pv. phaseolicola, which elicited an HR, blocked tumor formation by *A. tumefaciens*, while the *P. syringae* pv. phaseolicola mutant Hyr-25, which failed to elicit an HR, had little effect on tumor formation.

plant was placed in a 22°C growth chamber with a 16-h day and 27 W/m² photosynthetically active radiation light. The reactions at the injection sites were scored at 18, 24, 48, and 72 h after the injection of G50T. Browning and tissue collapse at the injection site were taken to represent the development of an HR. Bacterial strains which were capable of causing a greater than 24-h delay in or completely preventing the HR were considered able to inhibit the development of HR.

Wild-type strains of *A. tumefaciens* were capable of inhibiting the development of an HR in response to the injection of the wild-type toxin-producing strain 19304 of *P. syringae* pv. phaseolicola. However, the degree of inhibition of the HR was variable. Generally, the HR was not completely blocked but only delayed for various lengths of time. This may be due to the effects of the phaseolotoxin produced by *P. syringae* pv. phaseolicola on *A. tumefaciens*. Growth of *A. tumefaciens* on petri plates containing minimal medium was completely inhibited by the presence of phaseolotoxin-producing strains of *P. syringae* pv. phaseolicola. In

TABLE 3. Bacteria which inhibit the HR

Bacteria injected before <i>P. syringae</i> pv. phaseolicola	Inhibition of HR ^a
<i>A. tumefaciens</i> wild-type strains	
A6	+
C58	+
ACH5	+
15955	+
B6	+
1D1	+
Heat-killed <i>A. tumefaciens</i> A6	-
<i>R. meliloti</i>	
1027	-
1322	-
<i>E. coli</i> E6	-
<i>P. savastanoi</i> PB213	+

^a Ability to inhibit the HR elicited by *P. syringae* pv. phaseolicola in tobacco leaves by prior injection of the bacteria tested. +, No HR visible at 48 h; -, HR unaffected and first visible at 18 h.

order that the interpretation of the results of the experiments should not be complicated by the interaction of phaseolotoxin with *A. tumefaciens* or other bacteria, all experiments were carried out with the toxin-minus strain of *P. syringae* pv. phaseolicola (G50T Tox⁻). This mutant is capable of eliciting an HR in a fashion similar to the parent strain.

The number of viable bacteria per square centimeter of tobacco leaf was determined by punching disks of leaf tissue from within the inoculated area with a number 3 cork borer. The disks were ground in phosphate-buffered saline (11) in a Waring blender, and the number of viable bacteria was determined by plating on Luria agar for *A. tumefaciens* and on Luria agar containing neomycin (60 mg/liter) for G50T Tox⁻::Tn5.

RESULTS AND DISCUSSION

Effect of *P. syringae* pv. phaseolicola on tumor formation by *A. tumefaciens*. When *A. tumefaciens* C58 was inoculated alone into leaves of *B. diageomontiana*, 100% of the inoculated sites developed tumors (Table 2). When *P. syringae* pv. phaseolicola G50T Tox⁻ was inoculated prior to *A. tumefaciens*, only 6% of the inoculated sites developed tumors. The order of inoculation affected the ability of *P. syringae* pv. phaseolicola to inhibit tumor formation by *A. tumefaciens*. When *A. tumefaciens* was inoculated first and *P. syringae* pv. phaseolicola second, 88% of the inoculated sites developed tumors. When a mutant of *P. syringae* pv. phaseolicola which was unable to elicit an HR (Hyr-25) was used instead of wild-type bacteria, the effect of *P. syringae* pv. phaseolicola on tumor formation by *A. tumefaciens* was much reduced (Table 2 and Fig. 1). These results suggest that the elicitation of an HR may interfere with tumor formation by *A. tumefaciens*.

Effects of various bacterial species on development of the HR. Bacteria of species tested other than *A. tumefaciens* and *P. savastanoi* did not exhibit a detectable inhibition of the HR elicited by *P. syringae* pv. phaseolicola G50T Tox⁻ (Table 3). Neither the *A. tumefaciens* or *P. savastanoi* strains nor any of the nonphytopathogenic bacteria tested nor Luria broth elicited any visible response within 72 h when injected alone into tobacco leaves. *P. syringae* pv. phaseolicola G50T Tox⁻ elicited an HR which was visible by 24 h and resulted in complete tissue collapse, dessication, and browning of the area injected by 48 h. Prior injection of bacterial culture medium or *E. coli* or *Rhizobium meliloti* 1027 had no effect

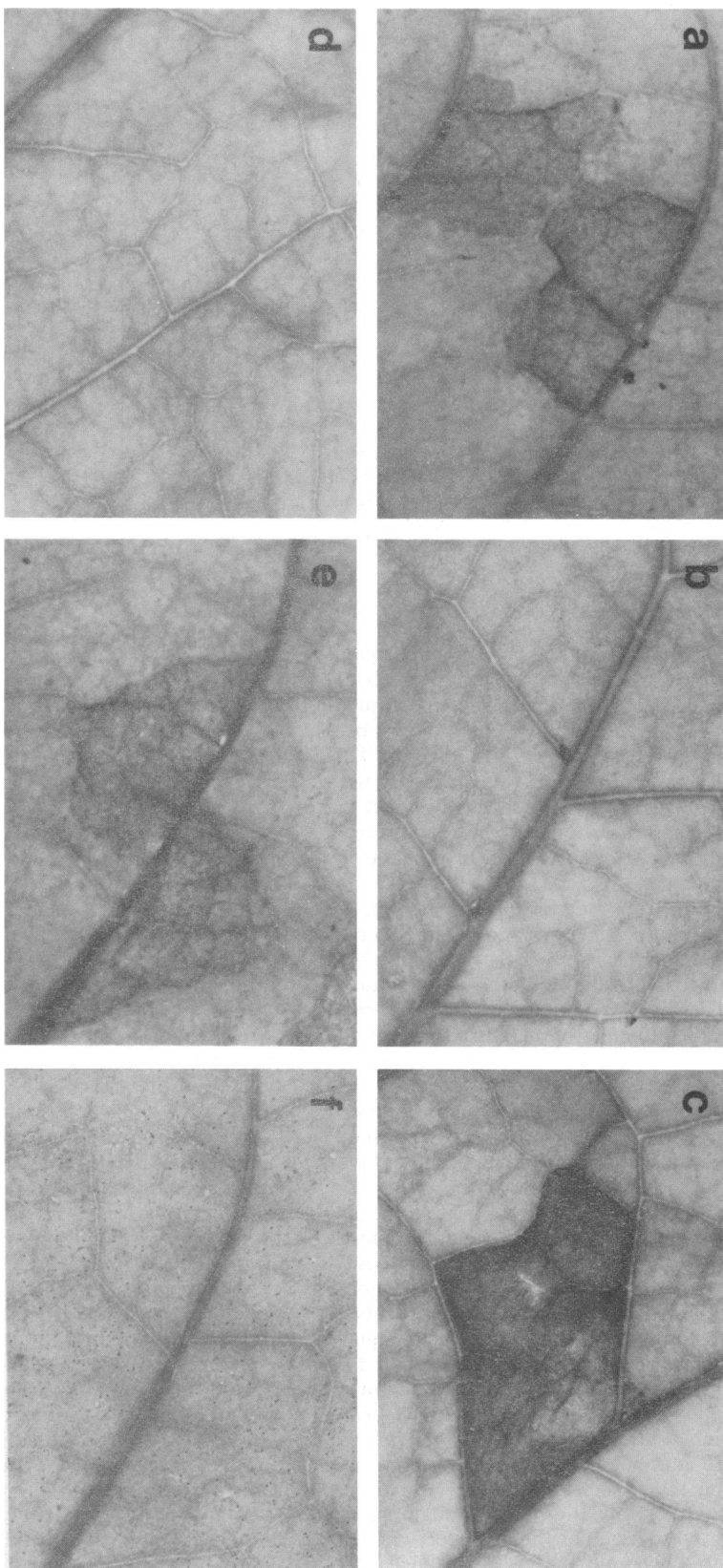


FIG. 2. Tobacco leaves photographed 2 days after the injection of bacteria. The bacteria injected were as follows: (a) *P. syringae* pv. phaseolicola G50 Tox⁻; (b) *A. tumefaciens* C58; (c) *A. tumefaciens* At121 followed by *P. syringae* pv. phaseolicola; (d) *A. tumefaciens* C38 followed by *P. syringae* pv. phaseolicola; (e) *A. tumefaciens* NT1 followed by *P. syringae* pv. phaseolicola; (f) *A. tumefaciens* NT1 (pE207) followed by *P. syringae* pv. phaseolicola. For an explanation of the bacterial strains used, see Tables 1 and 4.

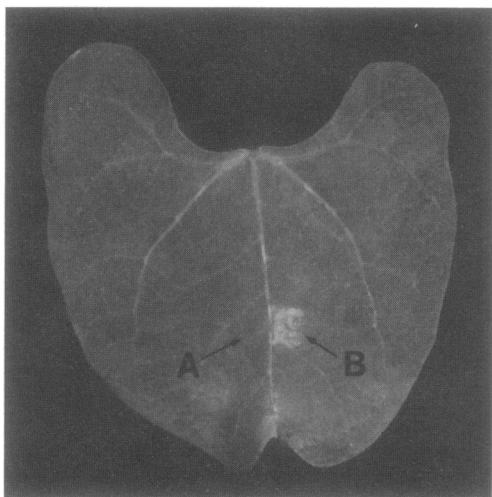


FIG. 3. Primary leaf of *Phaseolus vulgaris* UI34 injected with *A. tumefaciens* C58 at site A and with Luria broth at site B. Four hours later, both sites were injected with *P. syringae* pv. phaseolicola. The photograph was taken 24 h after the second injections. Note the absence of a visible HR at A and its presence at B. The injection of Luria broth or *A. tumefaciens* alone resulted in no visible reaction.

on the development of an HR elicited by the subsequent injection of *P. syringae* pv. phaseolicola G50T Tox⁻ in the same site in the tobacco leaf. However, the injection of wild-type strains of *A. tumefaciens* prevented the subsequent development of the HR (Table 3 and Fig. 2). This inhibition of the development of the HR was seen only with the injection of live *A. tumefaciens*. When the agrobacteria were heat killed prior to their injection, they were ineffective in preventing the development of the HR in response to *P. syringae* pv. phaseolicola.

Wild-type *A. tumefaciens* inhibited the development of the HR if they were injected at any time prior to the injection of *P. syringae* pv. phaseolicola. However, the inhibition was most reproducible if at least 2 h elapsed between injection of *A. tumefaciens* and of *P. syringae* pv. phaseolicola. In part, this may simply reflect the difficulty of making two injections at the same site in a tobacco leaf without allowing time for the first injection to dry completely.

Prior injection of *A. tumefaciens* also inhibited the development of the HR in response to *P. syringae* pv. phaseolicola G50T Tox⁻ in bean leaves of an incompatible cultivar (Fig. 3).

Growth of bacteria in tobacco leaf. One possible mechanism by which *A. tumefaciens* could inhibit the development of an HR in response to *P. syringae* pv. phaseolicola would be by inhibition of the growth of *P. syringae* pv. phaseolicola in leaf tissue. To examine this possibility, measurements of the number of viable *P. syringae* pv. phaseolicola per square centimeter of leaf tissue were made in sites which had received a prior injection of bacterial growth medium or wild-type *A. tumefaciens* (which inhibited the HR) or *A. tumefaciens* NT1 (which did not inhibit the HR; see below). In all three cases, the number of viable *P. syringae* pv. phaseolicola fell during the first 3 h. The *P. syringae* pv. phaseolicola then began to grow (Fig. 4). Although growth may have begun slightly sooner and been faster when *P. syringae* pv. phaseolicola was injected in the absence of *A. tumefaciens*, the differences observed were not statistically significant. No difference in bacterial growth was seen

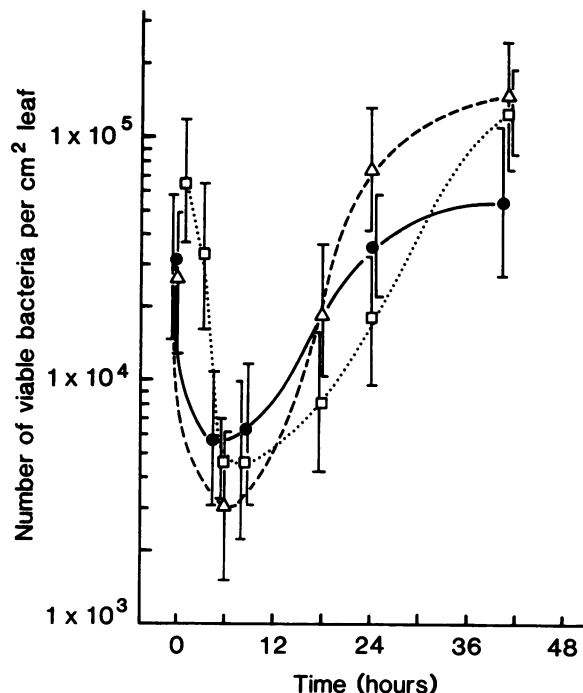


FIG. 4. Change in number of viable *P. syringae* pv. phaseolicola after injection into tobacco leaves at an initial inoculum of 3×10^4 to 6×10^4 bacteria per cm^2 of leaf tissue. —●—, Bacteria injected at a site injected 4 h previously with Luria broth; an HR was visible at these sites by 24 h. ---△---, Bacteria injected at a site injected 4 h previously with *A. tumefaciens* strain NT1; an HR was visible at these sites by 24 h. ···□···, Bacteria injected at a site injected 4 h previously with *A. tumefaciens* strain C58; no HR was visible at these sites at 48 h. Bars indicate standard deviations. Both *A. tumefaciens* strains remained constant at 2×10^5 bacteria per cm^2 of leaf tissue.

between *P. syringae* pv. phaseolicola injected into sites previously injected with *A. tumefaciens* strain C58 or NT1, although only the latter developed a visible HR. The number of viable *A. tumefaciens* remained constant in both cases at 2×10^5 bacteria per cm^2 of leaf. These results suggest that the mechanism by which *A. tumefaciens* inhibits the development of the HR does not involve inhibition of the growth of *P. syringae* pv. phaseolicola.

Interference with development of the HR by chromosomal mutants of *A. tumefaciens*. In order to determine which *A. tumefaciens* genes were required for inhibition of the HR, the ability of various previously characterized mutants of *A. tumefaciens* to inhibit the HR was examined. Wild-type strains A6, B6, 15955, Ach5, and C58 were able to inhibit the development of the HR (Table 3). Mutants of A6 which failed to make cellulose, such as Cel-12 (10), showed no diminution in their ability to inhibit the development of the HR. Mutants of C58 such as Att-C43 and Att-C539, which fail to attach to plant cells and are avirulent (12), were able to inhibit the development of the HR (Table 4). Thus, neither cellulose synthesis, virulence, nor the ability to bind to plant cells was required for the inhibition of the HR by *A. tumefaciens* (Table 4).

chvB mutants, unlike the chromosomal mutants described above, gave an intermediate response when tested for the ability to inhibit the development of the HR. The visible HR elicited by *P. syringae* pv. phaseolicola was reduced in intensity by prior injection of A1045, but an HR was always

TABLE 4. Effect of various mutations on the ability of *A. tumefaciens* to inhibit the HR

<i>A. tumefaciens</i> strain	Relevant characteristics	Inhibition of HR ^a
Cel-1	Cellulose-minus	+
Cel-12	Cellulose-minus	+
Att-C43	Attachment-minus, avirulent	+
A1038	<i>chvB</i> , attachment-minus, avirulent	+/-
A1045	<i>chvB</i> , attachment-minus, avirulent	+/-
A2507	<i>chvB</i> , attachment-minus, avirulent	+/-
C58	Wild type, nopaline pTi	+
NT1	C58 cured of pTi	-
ACH5	Wild type, octopine pTi	+
ACH5C3	ACH5 cured of pTi	-
At37	Carries pTi with <i>T</i> region deleted and <i>vir</i> region intact	-
At38	Carries pTi <i>T</i> region only	+
NT1(pE207)	Carries pTi <i>EcoRI</i> fragment 7 cloned in pRK2501	+
At91	pTi with Tn5 in <i>ocs</i>	+
At121	pTi with Tn3 in <i>tms-2</i>	-
At122	pTi with Tn5 in <i>tms-1</i>	-
At123	pTi with Tn5 in <i>tmr</i>	+

^a Ability to inhibit the HR elicited by *P. syringae* pv. phaseolicola in tobacco leaves by prior injection of the bacterial strain tested. +, No HR visible 48 h after the injection of *P. syringae* pv. phaseolicola. +/-, Reduced and delayed HR visible at 48 h (see Fig. 2), no HR visible at 18 h. -, No effect on the HR, which was first apparent at 18 h.

distinctly visible at sites where A1045 was injected first (Fig. 5). This was in contrast to the parent strain C58, which showed almost complete inhibition of the HR. *chvB* mutants are unable to make β -D-1,2-glucan, overproduce extracellular polysaccharide, fail to bind to plant cells, and are avirulent (5, 18).

Effects of the Ti plasmid on the ability to inhibit the HR. Bacteria which contain the Ti plasmid were compared with the same bacterial strain cured of the Ti plasmid to determine whether the presence of pTi is necessary for the inhibition of the HR. Wild-type strains C58 and Ach5 inhibited the development of the HR. When these strains were cured of pTi (strains NT1 and Ach5C3, respectively), they failed to inhibit the development of the HR (Table 4). Thus, some gene(s) on the Ti plasmid appears to be required for the ability to inhibit the HR.

In order to determine which region(s) of pTi contains the

gene(s) required for the inhibition of the HR, bacterial strains containing different functional regions of pTi were examined. Strain At37, which lacks the *T* region of pTi but contains an intact *vir* region (8), did not interfere with the development of the HR. Strain At38, which contains only the *T* region of pTi (7), completely blocked the development of the HR (Table 4). These results indicate that genes required for HR inhibition are probably located in the *T* region of pTi.

In order to determine which portion of the *T* region of pTi was required for the inhibition of the HR, the ability of strain NT1 containing a restriction fragment derived from pTi (*EcoRI* fragment 7 cloned in pRK2501) to inhibit the development of the HR was examined. Strain NT1 carrying this cloned pTi fragment was able to inhibit the HR as effectively as strain C58, which carries the entire pTi, suggesting that the region of pTi required for the inhibition of the HR was contained in *EcoRI* fragment 7.

A series of mutant strains containing transposon insertions in different sites in the *T* region were then examined to further localize the gene(s) involved in the inhibition of the HR. These strains, At121, At122, At123, and At91, are described in Tables 1 and 4. Only At121 and At122, with Tn5 insertions in the *tms* genes, failed to inhibit the HR (Table 4). Therefore, it would seem that functional *tms* genes are required for the inhibition of the HR. The *tms* genes code for the enzymes which synthesize the plant hormone auxin from tryptophan, suggesting that bacterial production of auxin may be involved in the inhibition of the HR. Studies of auxin production by *A. tumefaciens* suggest that Ti plasmid genes (possibly *tms* genes) are required (15). *tms* genes are expressed in the transformed plant (19). In addition, several laboratories have detected mRNA and protein products of *tms* genes in bacteria grown in the absence of plant cells (7, 9, 19, 20). Auxin production by *A. tumefaciens* is a complex topic which has been reviewed by R. O. Morris (15).

Analysis of the role of the production of plant hormones in the inhibition of the HR. The results described above suggested that bacterial production of auxin was involved in the inhibition of the HR. Auxin production by *A. tumefaciens* is reported to be reduced by the introduction of the plasmid pSa into the bacteria (3, 13). When the ability of *A. tumefaciens* strain SATC22 (which contains pSa) to inhibit the HR was examined, it was found that these bacteria failed to inhibit the development of the HR, although strain 1D1,

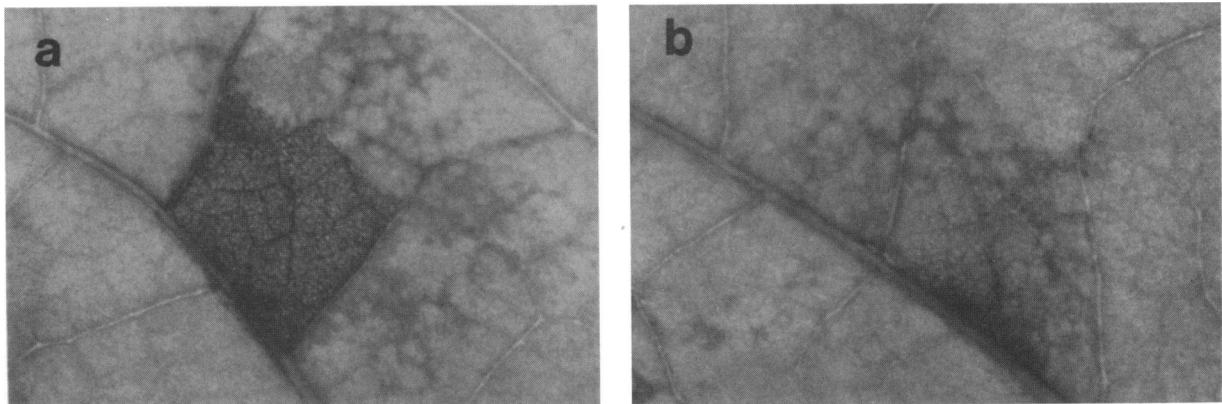


FIG. 5. Tobacco leaves photographed 2 days after the injection of bacteria. The bacteria injected were as follows: (a) *P. syringae* pv. phaseolicola; (b) *A. tumefaciens* A1045 followed by *P. syringae* pv. phaseolicola. Note that strain A1045 showed only a partial inhibition of the HR.

TABLE 5. Effect of ability of bacteria to produce plant hormones on their ability to inhibit the HR

Strain	Auxin production	Inhibition of HR ^a
<i>A. tumefaciens</i>		
C58	Wild type	+
15955	Wild type ^b	+
B6	Wild type	+
SATC22	Low ^b	-
<i>P. savastanoi</i>		
PB213	Wild type ^c	+
PB213-16 ^d	Wild type ^c	+/-
PB213-3 ^d	Low ^c	-

^a See Table 4, footnote a.

^b As determined by Chernin et al. (3).

^c As determined by Surico et al. (21).

^d This strain is reduced in cytokinin production (21).

which is the same strain lacking pSa, did inhibit the HR (Tables 1 and 5).

There is another plant pathogen which is known to produce phytohormones as part of the disease process, *P. savastanoi*. If the production of auxin is indeed involved in the inhibition of the HR, it is possible that wild-type auxin-producing *P. savastanoi* would be able to inhibit the development of the HR in a manner similar to *A. tumefaciens*. Wild-type *P. savastanoi* cells injected into tobacco leaves prior to the injection of *P. syringae* pv. phaseolicola were found to inhibit the development of the HR (Table 5). A mutant of *P. savastanoi* which is reduced in the production of the plant hormones auxin and cytokinin failed to inhibit the HR. Another mutant which is reduced in cytokinin production but which continues to produce auxin showed an intermediate ability to inhibit the HR (Fig. 6).

Plant hormone inhibition of an HR elicited by bacteria has been observed in cotton by Novacky (16), who found that cytokinins inhibited the development of the HR elicited by *Xanthomonas malvacearum*. In tobacco and carrot suspension cultures, the inclusion of auxins and cytokinins in the culture medium inhibited the development of the HR elicited by *P. syringae* pv. phaseolicola (14).

Phytohormone production may not be the only factor involved in the inhibition of the HR by *A. tumefaciens* and *P. savastanoi*. In neither case has the possible involvement of chromosomal genes been ruled out. In addition, when auxin (indoleacetic acid) at concentrations varying from 10^{-8} to 10^{-5} M was injected into leaves prior to *P. syringae* pv. phaseolicola, inhibition of the HR was not observed. This may be due to a failure of the auxin to persist in the leaf, or it may be due to a requirement for other factors in addition to auxin to inhibit the HR. Higher concentrations of auxin were toxic to the leaf tissue.

Previous work on early selective protection of tobacco leaves showed that substances produced when various *Pseudomonas* species (compatible, incompatible, or saprophytic) were injected into leaves could affect the growth and development of the HR by subsequently injected bacteria (2). The relationship of early selective protection and the inhibition of the HR by *A. tumefaciens* and *P. savastanoi* is unclear. In the experiments on early selective protection, the initially injected bacteria were flushed to the region surrounding the injected site before the inoculation of the incompatible bacterium. This technique was not possible with *A. tumefaciens*, which binds tightly to plant cells and is unchanged in number of viable cells per square centimeter of leaf after a second injection at the same site. Early selective protection

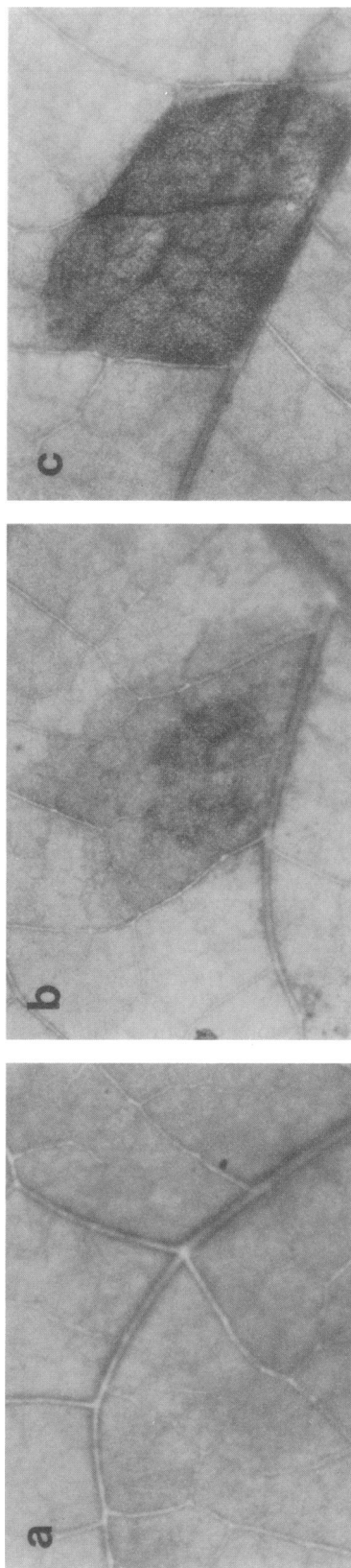


FIG. 6. Tobacco leaves photographed 2 days after the injection of bacteria. The bacteria injected were as follows: (a) *P. savastanoi* PB213 followed by *P. syringae* pv. phaseolicola; (b) *P. savastanoi* PB213-16 followed by *P. syringae* pv. phaseolicola; (c) *P. savastanoi* PB213-3 followed by *P. syringae* pv. phaseolicola. Note that strain PB213-16, which fails to produce cytokinins, showed a partial inhibition of the HR. Strain PB213-3, which fails to produce auxin and produces a reduced amount of cytokinins, failed to inhibit the HR.

was seen only in the region surrounding that which had been inoculated with the initial bacterium and not in the central region actually inoculated. Inhibition of the HR by *A. tumefaciens* was seen only in the region which had initially been inoculated with the bacteria. No reduction in the HR was seen beyond the borders of this region.

The results reported here suggest that the production of auxin and possibly cytokinin by tumor-inducing plant-pathogenic bacteria may inhibit the development of the HR by the plant. This inhibition could allow bacterial induction of tumors in the presence of other bacterial species which would ordinarily elicit an HR with sufficient speed that the host tissue would be dead before a tumor could be formed.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation grant DCB-84-16282, by U.S. Department of Agriculture grant 85-CRCR-1-1902, and by North Carolina Biotechnology grant ARIG 88-1012.

LITERATURE CITED

- Braun, A. C. 1962. Tumor inception and development in the crown gall disease. *Annu. Rev. Plant Physiol.* **13**:533-558.
- Burgyan, J., and Z. Klement. 1979. Early induced selective inhibition of incompatible bacteria in tobacco plants. *Phytopathol. Mediterr.* **18**:153-161.
- Chernin, L. S., E. V. Lobanok, V. V. Fomicheva, and N. A. Katel. 1984. Crown gall-suppressive IncW R plasmids cause a decrease in auxin production by *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* **195**:195-199.
- Deasey, M., and A. G. Matthyse. 1988. Characterization, growth and scanning electron microscopy of mutants of *Pseudomonas syringae* pv. *phaseolicola* which fail to elicit the hypersensitive response in host and non-host plants. *Physiol. Mol. Plant Pathol.* **33**:433-457.
- Douglas, C. J., W. Halperin, and E. W. Nester. 1982. *Agrobacterium tumefaciens* mutants affected in attachment to plant cells. *J. Bacteriol.* **152**:1265-1275.
- Garfinkel, D. J., and E. W. Nester. 1980. *Agrobacterium tumefaciens* mutants affected in tumorigenesis and octopine catabolism. *J. Bacteriol.* **144**:732-743.
- Gelvin, S. B., M. P. Gordon, E. W. Nester, and A. I. Aronson. 1981. Transcription of the *Agrobacterium* Ti plasmid in the bacterium and in crown gall tumors. *Plasmid* **6**:17-29.
- Hoekema, A., P. R. Hirsch, P. J. J. Hooykaas, and R. A. Schilperoort. 1983. A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature (London)* **303**:179-180.
- Janssens, A., G. Engler, P. Zambryski, and M. Van Montagu. 1984. The nopaline C58 T-DNA is transcribed in *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* **195**:341-350.
- Klement, Z. 1982. Hypersensitivity, p. 149-177. In M. S. Mount and G. H. Lacy (ed.), *Phytopathogenic prokaryotes*. Academic Press, Inc., New York.
- Matthyse, A. G. 1983. Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. *J. Bacteriol.* **154**:906-915.
- Matthyse, A. G. 1987. Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. *J. Bacteriol.* **169**:313-323.
- Matthyse, A. G. 1987. Effect of plasmid pSa and of auxin on attachment of *Agrobacterium tumefaciens* to carrot cells. *Appl. Environ. Microbiol.* **53**:2574-2582.
- Matthyse, A. G. 1987. A method for the bacterial elicitation of a hypersensitive-like response in plant cell cultures. *J. Microbiol. Methods.* **7**:183-191.
- Morris, R. O. 1986. Genes specifying auxin and cytokinin biosynthesis in phytopathogens. *Annu. Rev. Plant Physiol.* **37**:509-538.
- Novacky, A. 1972. Suppression of the bacterially induced hypersensitive reaction by cytokinins. *Physiol. Plant Pathol.* **2**:101-104.
- Patil, S. S., A. C. Hayward, and R. Emmons. 1974. An ultraviolet-induced nontoxic mutant of *Pseudomonas phaseolicola* of altered pathogenicity. *Phytopathology* **64**:590-595.
- Puvanesarajah, V. F., M. Schell, G. Stacey, C. J. Douglas, and E. W. Nester. 1985. Role for 2-linked- β -D-glucan in the virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* **164**:102-106.
- Schroeder, G., W. Klipp, A. Hildebrand, R. Ehring, C. Koncz, and J. Schröder. 1983. The conserved part of the T-region in Ti-plasmids expresses four proteins in bacteria. *EMBO J.* **2**:403-409.
- Schroeder, G., S. Waffenschmidt, E. W. Weiler, and J. Schröder. 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. *Eur. J. Biochem.* **138**:387-391.
- Surico, G., L. Comai, and T. Kosuge. 1984. Pathogenicity of strains of *P. syringae* pv. *savastanoi* and their IAA-deficient mutants on olive and oleander. *Phytopathology* **74**:490-493.
- Sykes, L. C., and A. G. Matthyse. 1986. Time required for tumor induction by *Agrobacterium tumefaciens*. *Appl. Environ. Microbiol.* **52**:597-598.
- Virts, E. L., and S. B. Gelvin. 1985. Analysis of transfer of tumor-inducing plasmids from *Agrobacterium tumefaciens* to petunia protoplasts. *J. Bacteriol.* **162**:1030-1038.