

Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase

Xiaoyan Tang,* Reid D. Frederick,* Jianmin Zhou, Dennis A. Halterman, Yulin Jia, Gregory B. Martin†

Resistance to bacterial speck disease in tomato occurs when the Pto kinase in the plant responds to expression of the avirulence gene *avrPto* in the *Pseudomonas* pathogen. Transient expression of an *avrPto* transgene in plant cells containing *Pto* elicited a defense response. In the yeast two-hybrid system, the Pto kinase physically interacted with AvrPto. Alterations of AvrPto or Pto that disrupted the interaction in yeast also abolished disease resistance in plants. The physical interaction of AvrPto and Pto provides an explanation of gene-for-gene specificity in bacterial speck disease resistance.

Disease resistance in many plant-pathogen interactions results from the expression of a resistance (*R*) gene in the plant and a corresponding avirulence (*avr*) gene in the pathogen (1) and is often associated with the rapid, localized cell death of the hypersensitive response (HR). *R* genes that respond to specific bacterial, fungal, or viral pathogens have been isolated from a variety of plant species and several appear to encode cytoplasmic proteins (2–5). It has been unclear how such proteins could recognize an extracellular pathogen.

Sequence analysis of over 30 bacterial *avr* genes has generated little insight into the recognition process (6). Some bacterial pathogens of mammals use a protein secretion system, the type III pathway, to inject virulence proteins directly into the host cell (7). Components of a type III pathway are also encoded by the *Hrp* genes in many bacterial pathogens of plants, including *Pseudomonas* species (8). Thus phyto-bacterial pathogens might directly introduce Avr proteins into plant cells. A report that *avrB* from *Pseudomonas syringae* pv. *glycinea* elicits an *R* gene-dependent HR when expressed within plant cells supports this model (9).

We examined the interaction between tomato and the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. Research with this system has led to the isolation of the bacterial *avrPto* gene and the tomato *Pto* gene that, when expressed in the corresponding organisms, result in resistance to bacterial speck disease (2, 10, 11). Sequence analysis of *avrPto* has not revealed its function (11). The *Pto* gene encodes a serine-threonine kinase and is a member of a clustered gene family that also includes

the *Fen* gene (2, 12, 13). The amino acid sequence of *Fen* kinase is 87% similar to *Pto*. *Fen* confers sensitivity to the insecticide fenthion (12). Other components of this signaling pathway in tomato include the *Prf* and *Pti1* genes. *Prf* has similarities to a broad class of *R* gene products in that it contains leucine-rich repeats and a nucleotide binding site (14). *Pti1* is a serine-threonine kinase probably acting downstream of *Pto* (15). We report here that the bacterial AvrPto protein directly interacts with the plant *Pto* kinase.

We used an *Agrobacterium*-mediated transient gene expression assay (16) to test if AvrPto protein could induce an HR when expressed inside plant cells (Fig. 1). Tobacco plants overexpressing the tomato *Pto* gene were used for this assay because they

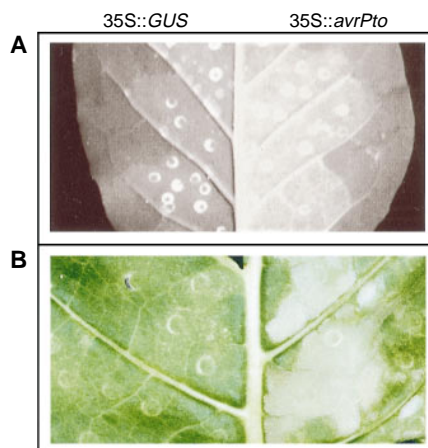


Fig. 1. Expression of *avrPto* in tobacco leaves. *Agrobacterium tumefaciens* strain EHA105 containing a 35S::*GUS* construct (left) or a 35S::*avrPto* construct (right) was injected into leaves of tobacco line W-38 expressing a 35S::*Pto* transgene (17). (A) Accumulation of ultraviolet-fluorescent compounds at 32 hours after infiltration. (B) Development of the HR at 48 hours after infiltration.

develop an enhanced HR specifically in response to *Pseudomonas syringae* pv. *tabaci* expressing *avrPto* (17). The *avrPto* gene under control of the cauliflower mosaic virus 35S promoter was introduced into *Agrobacterium* EHA105, which was subsequently infiltrated into fully expanded tobacco leaves (16). Ultraviolet-stimulated fluorescence was observed 32 hours after infiltration, indicating the accumulation of phenolic compounds associated with disease resistance (Fig. 1A). An HR appeared approximately 48 hours after injection (Fig. 1B). Neither fluorescence nor an HR occurred in leaves injected with either EHA105 containing pBI121 alone or with *Agrobacterium* strain A136 containing the 35S::*avrPto* construct but lacking the *Ti* plasmid (18). Thus, AvrPto protein induces a defense response when introduced directly into plant cells expressing the *Pto* gene (Fig. 1, A and B).

Because the *Pto* kinase confers recognition specificity in bacterial speck resistance, we tested whether *Pto* and AvrPto physically interact in the yeast two-hybrid system (19) (Fig. 2). Neither AvrPto nor *Pto* expressed individually activated the *lacZ* reporter gene in the two-hybrid system (Fig. 2). However, expression of both AvrPto and *Pto* in the same yeast cell activated the *lacZ* gene, demonstrating interaction of these two proteins (Fig. 2). Co-expression of AvrPto with kinases encoded by the recessive *pto* allele (20), the *Fen* gene, or with a mutant *Pto* protein that is unable to autophosphorylate (*Pto*[K69Q]; 13) did not activate *lacZ* (Fig. 2). The physical interaction of AvrPto with *Pto* suggests that AvrPto serves as a bacterial signal molecule and that *Pto* serves as the corresponding receptor.

To determine if particular regions of *Pto* are required for interaction with AvrPto, we constructed a series of chimeric proteins each consisting of different portions of *Pto* and *Fen* (Fig. 3A) (21). All *Pto*-*Fen* chimeric proteins possess kinase activity as determined by *in vitro* autophosphorylation assays [we were unable to express chimeric

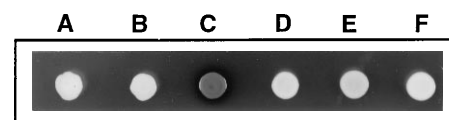


Fig. 2. Interaction of AvrPto with *Pto*. The LexA two-hybrid system (19) was used to test possible interaction of AvrPto with *Pto* and other closely related kinases. In all cases the *avrPto* gene was introduced on pJG4-5, and the other genes were introduced on pEG202. Yeast strains were grown at 30°C for 2 days on galactose, X-Gal complete minimal medium (19). Yeast strains contain: (A) AvrPto, (B) *Pto*, (C) AvrPto/*Pto*, (D) AvrPto/*Pto*, (E) AvrPto/*Pto*(K69Q), or (F) AvrPto/*Fen*.

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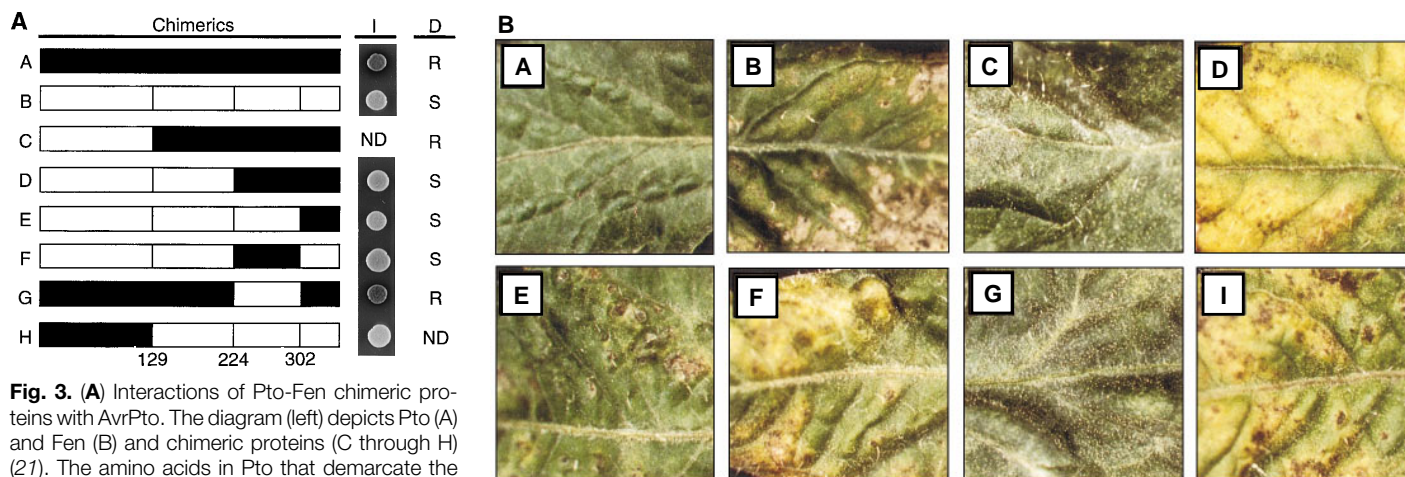


Fig. 3. (A) Interactions of Pto-Fen chimeric proteins with AvrPto. The diagram (left) depicts Pto (A) and Fen (B) and chimeric proteins (C through H) (21). The amino acids in Pto that demarcate the junction points between portions from Pto (in black) and portions from Fen (white) are shown at the bottom. EGY48 yeast cells containing AvrPto (in pJG4-5) and the various Pto-Fen chimeric proteins (in pEG202) were grown at 30°C for 2 days on galactose, X-Gal complete minimal medium (center). Similar expression of each protein in yeast was verified by protein immunoblots (Western) (32). I, Interaction assays in the two-hybrid system. D, disease responses of the corresponding transgenic MoneyMaker plants inoculated with avirulent *P. syringae tomato* strain T1(pPtE6) (12). R, resistant; S, susceptible; and ND, not determined.

(B) Disease responses of transgenic tomato plants containing the Pto-Fen chimeric constructs. Leaves of primary transformants (24) were inoculated by dipping into a solution of avirulent *P. syringae tomato* strain T1(pPtE6) (4×10^7 cfu/ml; 2). Photographs were taken 5 days after inoculation. The leaves shown are from plants containing the following transgenes under transcriptional control of the CaMV 35S promoter: **(A)** Pto, **(B)** Fen, and **(C to G)** chimeric constructs (C), (D), (E), (F), and (G). [Chimeric construct **(H)** was not transformed into MoneyMaker]. A leaf from a nontransgenic MoneyMaker plant is shown in **(I)**.

construct C in *Escherichia coli* (13, 22)]. In the two-hybrid system, AvrPto specifically interacted with chimera G (23) (Fig. 3A). Comparison of chimera G with the other chimeras implicated a region in Pto from amino acids 129 to 224 that is required for interaction with AvrPto. We also generated stable transgenic tomato plants expressing each of the *Pto-Fen* constructs to examine the requirement of different regions of the Pto protein for disease resistance (24) (Fig. 3B). Only chimeric genes C and G conferred resistance in tomato to the avirulent pathogen whereas the other chimeric genes conferred sensitivity to fenthion (Fig. 3B) (22). Thus a 95-amino acid stretch of the Pto kinase is involved in pathogen recognition and also forms a part of the interaction

site with the AvrPto protein.

Several carboxy-terminal deletions of AvrPto were assayed for interaction with Pto in the two-hybrid system (Fig. 4A) (25). Deletions CΔ12 and CΔ25, lacking 12 and 25 amino acids, respectively, from the carboxy terminus interacted with Pto whereas the others did not (Fig. 4A).

The *avrPto* deletions were also introduced into two *P. syringae* pathovars, *tomato* and *tabaci*, and inoculated onto tomato and tobacco plants to assess their effects on the HR and disease symptoms (26). Only the two AvrPto deletions that interacted with Pto in the two-hybrid system, CΔ12 and CΔ25, induced both an HR and disease resistance in a Pto-dependent manner (Table 1). Disease resistance was quantified by

measuring bacterial growth after inoculation of Pto-transgenic tobacco leaves with 10^5 cfu/ml (17) (Fig. 4B). Expression of CΔ41 in *P. syringae tabaci* did not affect bacterial growth in leaves, whereas expression of CΔ12 and CΔ25 reduced the final bacterial populations by 15- and 6-fold, respectively, compared to a *P. syringae tabaci* strain lacking *avrPto* (Fig. 4B) (27). Therefore, the ability of AvrPto to interact with Pto in the two-hybrid system correlates with its ability to elicit disease resistance in plants.

Genetic analysis of many plant-pathogen associations has supported a model for direct interaction between R gene products and *avr* gene products (28). However, the inability to detect secretion of bacterial Avr

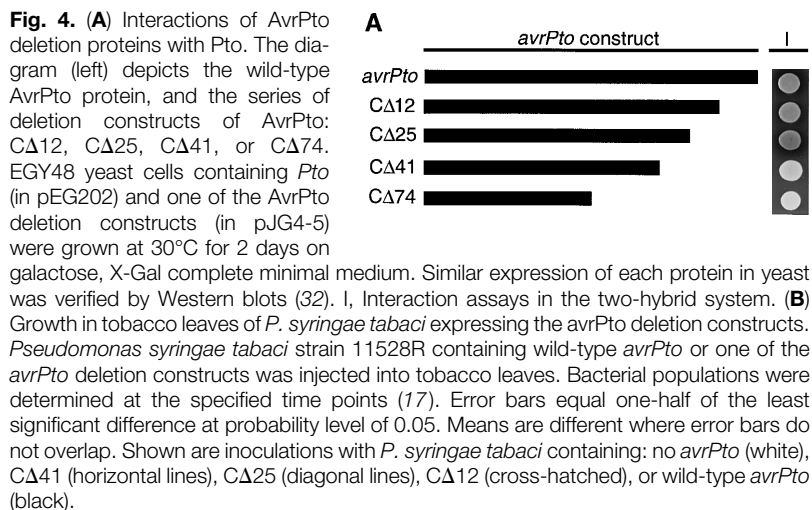


Fig. 4. (A) Interactions of AvrPto deletion proteins with Pto. The diagram (left) depicts the wild-type AvrPto protein, and the series of deletion constructs of AvrPto: CΔ12, CΔ25, CΔ41, or CΔ74. EGY48 yeast cells containing Pto (in pEG202) and one of the AvrPto deletion constructs (in pJG4-5) were grown at 30°C for 2 days on galactose, X-Gal complete minimal medium. Similar expression of each protein in yeast was verified by Western blots (32). I, Interaction assays in the two-hybrid system. **(B)** Growth in tobacco leaves of *P. syringae tabaci* expressing the *avrPto* deletion constructs. *Pseudomonas syringae tabaci* strain 11528R containing wild-type *avrPto* or one of the *avrPto* deletion constructs was injected into tobacco leaves. Bacterial populations were determined at the specified time points (17). Error bars equal one-half of the least significant difference at probability level of 0.05. Means are different where error bars do not overlap. Shown are inoculations with *P. syringae tabaci* containing: no *avrPto* (white), CΔ41 (horizontal lines), CΔ25 (diagonal lines), CΔ12 (cross-hatched), or wild-type *avrPto* (black).

Table 1. Analysis of *avrPto* deletions in *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci* inoculated on tomato or tobacco, respectively. The ability to elicit the hypersensitive response was assayed by infiltrating 10⁸ colony-forming units per milliliter into tomato or tobacco leaves. The ability of the *Pseudomonas* strains to cause disease symptoms was assayed by infiltrating tobacco leaves with 10⁵ cfu/ml or dipping tomato leaves into 10⁷ cfu/ml. Tomato lines analyzed were near isogenic cultivars Rio Grande-PtoR (*Pto/Pto*) and Rio Grande (*pto/pto*). Tobacco line was Wisconsin-38 containing a 35S::*Pto* transgene (17). + indicates a hypersensitive response or disease symptoms were observed; – indicates no hypersensitive response or disease symptoms were observed; N.D., not determined.

Plant Genotype	<i>P. syringae</i> pv. <i>tomato</i>				<i>P. syringae</i> pv. <i>tabaci</i>	
	Hypersensitive response*		Disease symptoms†		Hypersensitive response	Disease symptoms
	Tomato		Tomato		Tobacco	Tobacco
	<i>Pto/Pto</i>	<i>pto/pto</i>	<i>Pto/Pto</i>	<i>pto/pto</i>	35S:: <i>Pto</i>	35S:: <i>Pto</i>
Construct						
<i>avrPto</i>	+	–	–	+	+	–
CΔ12	+	–	–	+	+	–
CΔ25	+	–	–	+	+	–
CΔ41	–	–	+	+	–	+
CΔ74	–	–	+	+	N.D.	N.D.
None	–	–	+	+	–	+

*Hypersensitive response appeared as tissue collapse of the infiltrated area and was scored 14 hours after infiltration. lesions and in tobacco as dark, water-soaked regions. Symptoms were scored 5 days after inoculation.

†Disease symptoms in tomato appeared as small necrotic

proteins and the apparent cytoplasmic location of several R gene products seemed to preclude such a mechanism (2, 3, 29). Our results support such a model for bacterial speck resistance and suggest functional implications of the AvrPto-Pto interaction. The interaction of AvrPto with Pto, perhaps anchored to the plasma membrane by Prf, may stimulate Pto kinase activity and trigger a phosphorylation cascade. Alternatively, AvrPto may facilitate dimerization and cross-phosphorylation between Pto molecules. Finally, AvrPto might participate in a protein complex involving other proteins, including Prf, that activates the Pto signaling pathway.

How universal is this mode of recognition in plant pathogen interactions? Gene products that confer resistance to *Pseudomonas* species (2, 3, 14), and to a fungal pathogen and an intracellular viral pathogen (4) appear to be cytoplasmic. Direct protein-protein interactions within the plant cell would be consistent with the gene-for-gene specificity seen in these associations. However, not all R gene products are alike. Pto, for example, is a cytoplasmic protein kinase (2). The other R genes, and Prf, encode proteins containing leucine-rich repeats and in some cases a nucleotide binding site (3–5, 14). Certain R gene products appear to have extracellular domains and may be involved in protein-protein interactions that are external to the plant cell (5).

Bacterial pathogens of plants and mammals share common components for the type III protein secretion pathway whereby virulence factors are delivered directly into host cells (7). In some *Pseudomonas* species, the same virulence factors are employed against both plants and animals (30). *Yer-*

sinia pseudotuberculosis, a mammalian enteropathogen, disrupts host signal transduction by introducing a serine-threonine kinase and a phosphatase into the mammalian host cell (31). We have shown that a signal transduction pathway that leads to disease resistance in plants is also the target of a bacterial pathogen signal molecule; however, the result in this instance is recognition of the pathogen. Conservation of virulence mechanisms among plant and mammalian bacterial pathogens suggests that similar disease resistance mechanisms may have also evolved in these taxonomic kingdoms.

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- The DNA sequence of the *avrPto* gene was ligated into the *Xba*I and *Sac*I sites of pBI121 and introduced into *A. tumefaciens* EHA105 and A136. *Agrobacterium* cells were inoculated into liquid AB medium supplemented with 50 mg/L kanamycin and 0.2 mM acetosyringone and grown at 30°C for 1 day. Cells were washed twice, resuspended in 10 mM MgCl₂ to a final concentration of 10⁸ cfu/ml, and injected into tobacco leaves.
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- Oligonucleotide primers containing restriction enzyme sites were designed from conserved regions of the *Pto* and *Fen* genes that allowed the three carboxy-terminal regions to be amplified by PCR (22). PCR products were cleaved with appropriate restriction enzymes and ligated together to create chimeric *Pto-Fen* constructs (22). A conserved *Bgl* II site in both *Pto* and *Fen* allowed for reciprocal amino terminal exchanges at amino acid 129. All constructs were verified by sequencing.
- R. Frederick and G. Martin, unpublished results.
- Chimeric *Pto-Fen* gene constructs were cloned into pEG202 with the use of either Eco RI (*Fen* carboxy-terminal region) or Eco RI–Bam HI (*Pto* carboxyl terminal region) sites and introduced into yeast EGY48 containing the *avrPto* gene in pJG4-5 (19).
- Chimeric *Pto-Fen* gene constructs were cloned into pBI121 and the plasmids were introduced into *A. tumefaciens* EHA105. Chimeric constructs were transferred into tomato cultivar MoneyMaker with the use of *Agrobacterium*-mediated transformation. Transgenic status of plants was verified by probing genomic DNA blots with a *Pto* gene probe and with the *nptII* gene from pBI121 (22).
- Full-length *avrPto* and the *avrPto* deletions were ligated into the Eco RI and Xho I sites of pJG4-5 (19). Constructs were introduced into the yeast strain EGY48 containing the *Pto* gene in pEG202 (19). All constructs were verified by sequencing.
- avrPto* deletions were cloned into pPIE6 (11) and introduced into *P. syringae* tomato T1 or *P. syringae* *tabaci* 11528R by triparental mating. Transconjugants were verified by DNA blot analysis.
- The reduced avirulence activity of the mutant AvrPto proteins might be due to less efficient secretion of

- AvrPto from the *Pseudomonas* cell. To examine this, *avrPto* deletions CΔ25, CΔ41, and CΔ74 were placed into pBI121 and tested with the *Agrobacterium* transient assay. *Agrobacterium* EHA105 containing *avrPto* induced an HR in 2 days, whereas EHA105 containing the *avrPto* deletion CΔ25 induced an HR after 4 days; the other deletions did not elicit an HR (X. Tang and G. Martin, unpublished results). This suggests that the carboxyl terminal 25 amino acids of AvrPto are not required for secretion from the bacterial cell; this portion of AvrPto may serve as an activation domain, interact with other components in the signaling pathway, or have a role in AvrPto stability.
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 33. Levels of protein expression were determined with the use of antibody to LexA (a gift from E. Golemis) for LexA fusion proteins (in pEG202) and antibody to the hemagglutinin (HA) epitope tag (Boehringer-Mannheim) for the AvrPto::HA fusion protein (in pJG4-5).
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Molecular Basis of Gene-for-Gene Specificity in Bacterial Speck Disease of Tomato

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Transient expression of the *Pseudomonas syringae* avirulence gene *avrPto* in plant cells resulted in a *Pto*-dependent necrosis. The AvrPto avirulence protein was observed to interact directly with the Pto resistance protein in the yeast two-hybrid system. Mutations in the *Pto* and *avrPto* genes which reduce in vivo activity had parallel effects on association in the two-hybrid assay. These data suggest that during infection the pathogen delivers AvrPto into the plant host cell and that resistance is specified by direct interaction of Pto with AvrPto.

In plants, resistance to a variety of pathogens is determined by the action of complementary pairs of resistance (*R*) genes in the host and avirulence (*avr*) genes in the pathogen. These gene-for-gene interactions have been observed between plants and a diverse array of pathogens, including viruses, bacteria, fungi, nematodes, and insects (1). From genetic analysis it has been proposed that *R* genes recognize an elicitor produced directly or indirectly by the pathogen's *avr* gene, which leads to a resistance response in the infected plant (2).

Bacterial speck disease of tomato is caused by *Pseudomonas syringae* pv. *tomato*

(*Pst*). In tomato, resistance to strains of *Pst* that contain the *avr* gene *avrPto* is conferred by the *Pto* gene (3). The *Pto* locus encodes a family of related serine-threonine kinases. Among these, *Fen* is active in a parallel pathway that confers sensitivity to the insecticide fenthion (4).

R and *avr* proteins may interact directly, thereby activating plant defenses. For the protein product of *Pto*, this binding would likely occur intracellularly because of its predicted cytoplasmic localization. The activity of many *avr* genes, including *avrPto*, depends on an *hrp* secretion pathway which is similar to the type III secretory systems of

Yersinia, *Shigella*, and *Salmonella* (5). These pathogens translocate a set of virulence proteins into host cells. Therefore, we considered the possibility that the bacterial AvrPto protein moves across the plant cell wall and plasma membrane where it directly interacts with the tomato Pto protein.

Evidence indicating that AvrPto acts inside the plant cell was obtained by transiently expressing *avrPto* in transgenic *Nicotiana benthamiana* plants transformed with *Pto* (6) (Fig. 1). This resulted in necrosis similar to the *Pto*-mediated HR elicited by *P. syringae* expressing *avrPto* and indicated that AvrPto was active within the plant cell. Deletion of 30 amino acids from the COOH-terminus of AvrPto did not eliminate this activity, whereas deletion of 59 amino acids destroyed activity. Activity of the deletion derivatives in the transient expression assays correlated with biological activity in *P. syringae*. These results suggested that the products of the *avr* and *R* genes may interact directly.

We employed the yeast two-hybrid system to directly test this hypothesis (7). *Pto*, *Fen*, and *avrPto* coding sequences were expressed as fusions to GAL4 DNA binding (BD) and transcriptional activating (AD) domains. Reciprocal combinations of BD and AD fusions were tested for β -galactosidase reporter gene activity in yeast. Interaction was only observed when the BD::Pto and AD::AvrPto fusions were coexpressed (Fig. 2). Controls did not show any interaction. Furthermore, no interaction was detected between BD::Fen and AD::AvrPto (Figs. 2 and 3A).

To test the biological relevance of the interaction, inactive alleles of *Pto* and *avrPto* were tested. Three inactive *Pto* alleles, *pto6*, *pto7* and *pto11*, were previously identified through mutagenesis of resistant tomato plants (8). Sequence analysis revealed single amino acid changes in each mutant allele (Fig. 3B). The mutant *Pto* sequences showed no detectable interaction with AvrPto in yeast. Thus, mutant alleles that confer susceptibility to *Pst* also fail to interact with AvrPto in the two-hybrid system. The two deletions of AvrPto tested in

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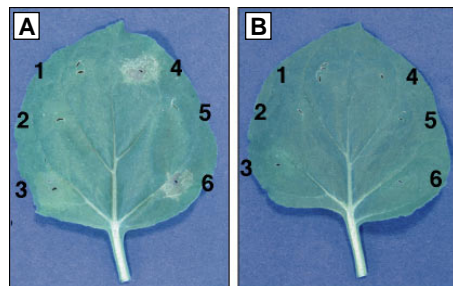
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Fig. 1. *Agrobacterium tumefaciens* strain A281 (sites 3 to 6) or the nontumorigenic strain C58C1 (sites 1 and 2) containing *avrPto* gene constructs (10) were infiltrated into leaves of a *Pto* transformed plant (A) or leaves of untransformed *N. benthamiana* (B). Expression of *avrPto* (sites 1 and 4), and *avrPto* deletions of 30 (site 6), and 59 (site 3) amino acids off the COOH-terminus was controlled by the CaMV 35S viral promoter in a binary plant transformation vector. An *avrPto* construct (pPtE6) lacking left and right T-DNA borders or a plant promoter (11) was also infiltrated (sites 2 and 5). Leaves were photographed 3 days after inoculation.



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