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Tomato Mutants Altered in Bacterial Disease Resistance Provide Evidence for a New Locus Controlling Pathogen Recognition

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We have employed a genetic approach to study the resistance of tomato to the phytopathogenic bacterium *Pseudomonas syringae* pv *tomato*. Resistance to *P. s. tomato* depends upon expression of the *Pto* locus in tomato, which encodes a protein with similarity to serine/threonine protein kinases and recognizes pathogen strains expressing the avirulence gene *avrPto*. Eleven tomato mutants were isolated with altered resistance to *P. s. tomato* strains expressing *avrPto*. We identified mutations both in the *Pto* resistance locus and in a new locus designated *Prf* (for Pseudomonas resistance and fenthion sensitivity). The genetic approach allowed us to dissect the roles of these loci in signal transduction in response to pathogen attack. Lines carrying mutations in the *Pto* locus vary 200-fold in the degree to which they are susceptible to *P. s. tomato* strains expressing *avrPto*. The *pto* mutants retain sensitivity to the organophosphate insecticide fenthion; this trait segregates with *Pto* in genetic crosses. This result suggested that contrary to previous hypotheses, the *Pto* locus controls pathogen recognition but not fenthion sensitivity. Interestingly, mutations in the *prf* locus result in both complete susceptibility to *P. s. tomato* and insensitivity to fenthion, suggesting that *Prf* plays a role in tomato signaling in response to both pathogen elicitors and fenthion. Because *pto* and *prf* mutations do not alter recognition of *Xanthomonas campestris* strains expressing *avrBsP*, an avirulence gene recognized by all tested tomato cultivars, *Prf* does not play a general role in disease resistance but possibly functions specifically in resistance against *P. s. tomato*. Genetic analysis of F_2 populations from crosses of *pto* and *prf* homozygotes indicated that the *Pto* and *Prf* loci are tightly linked.

INTRODUCTION

In a wide variety of plant species, resistance to pathogens is frequently expressed as a rapid localized necrosis of host tissue termed the hypersensitive response (HR). The HR involves a series of dramatic changes in cell physiology and is associated with severe restriction of pathogen growth (Klement, 1982). Physiological events associated with the HR include an increase in lytic activites, an oxidative burst, alterations in cell wall metabolism, and phospholipid turnover (Dixon and Lamb, 1990). The ability to express the HR is under the genetic control of plant disease resistance loci (Dixon and Lamb, 1990), and it has been hypothesized that resistance gene products may function in signal transduction pathways linking pathogen recognition to expression of disease resistance (Keen, 1990).

A major barrier to testing this hypothesis has been the lack of information concerning the structure of disease resistance loci or their functions in governing disease resistance. For example, until recently, only one such locus had been cloned: the *HM1* locus from maize. The *HM1* locus specifically controls resistance to race 1 isolates of the fungal pathogen *Cochliobolus carbonum* (Johal and Briggs, 1992). Because the *HM1* gene product enzymatically inactivates the fungal HC-toxin (Meeley et al., 1992), other resistance genes can be expected to exhibit modes of action qualitatively different from *HM1*. Specificity of resistance loci for a subset of pathogen isolates is commonly observed and is based on the presence of cognate genes in the pathogen termed avirulence genes (Long and Staskawicz, 1993). Disease resistance results when interacting organisms express a matching pair of resistance and avirulence loci, and susceptibility results if either the resistance locus or the avirulence locus is absent. In this way, both host and pathogen exert genetic control over the outcome of their interaction.

In contrast to other biological processes in plants (Okada and Shimura, 1992), a genetic approach has rarely been employed in the study of plant disease resistance. A genetic

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approach should provide mutant lines useful in the cloning of disease resistance loci, analysis of their structures, and elucidation of their functions in pathogen recognition and expression of the HR. One example has already been reported in Arabidopsis, in which isolation of mutant lines has led to the identification of the *RPS2* locus governing resistance to *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2* (Kunkel et al., 1993; Yu et al., 1993). Mutational analysis may also allow us to identify other plant factors with which resistance gene products cooperate in plant signaling in response to pathogen recognition. For example, through mutagenesis, two loci have recently been identified in tomato that modify expression of resistance to *Cladosporium fulvum* mediated by the *Cf-9* resistance locus (Hammond-Kosack et al., 1994). In another study, Jorgensen (1988) undertook genetic dissection of barley resistance to *Erysiphe graminis*, which is governed by the *Ml-a12* resistance locus. Although 22 of 25 isolated mutations mapped to the *Ml-a12* locus, three mapped to one or more "modifier" loci (Jorgensen, 1988), supporting the concept that *Mla-12* acts cooperatively with other barley loci to effect successful pathogen defense.

The phytopathogenic bacterium *P. s.* pv *tomato* causes bacterial speck disease of tomato, which is responsible for significant yield losses annually, especially in regions where cool, moist weather is prevalent early in the growing season (Yunis et al., 1980). In tomato, resistance to *P. s. tomato* strains that express the avirulence gene *avrPto* (Ronald et al., 1992) is governed by the incompletely dominant *Pto* resistance locus on chromosome 5 (Pitblado et al., 1984; Carland and Staskawicz, 1993; Martin et al., 1993a). *Pto* was introgressed into *Lycopersicon esculentum* cultivars from the sexually compatible resistant species *L. pimpinellifolium* (Pitblado and MacNeill, 1983). Morphological and molecular markers tightly linked to *Pto* have been identified (Carland and Staskawicz, 1993; Martin et al., 1993a), and molecular cloning of the *Pto* gene has recently been accomplished using a positional cloning strategy (Martin et al., 1993b). *Pto* is a member of a clustered family of five to seven genes and is predicted to encode a serine/threonine protein kinase (Martin et al., 1993b), suggesting a role in a signal transduction pathway. In addition, the *avrPto* avirulence gene that corresponds to *Pto* has been cloned from *P. s. tomato* (Ronald et al., 1992). The *avrPto* gene encodes a mostly hydrophilic protein of 18.3 kD that shows no similarity to entries in protein sequence data bases, and its expression is regulated by carbon source and *P. s. tomato hrp* genes that control pathogenicity and induction of the HR (Salmeron and Staskawicz, 1993).

A particularly interesting feature of the tomato–*P. s. tomato* system is the sensitivity of tomatoes carrying the *Pto* locus to the organophosphate insecticide fenthion (Laterrot and Philouze, 1985). On tomatoes carrying *Pto*, fenthion causes a necrotic reaction that mimics the plant HR in response to *P. s. tomato* strains expressing *avrPto* (Laterrot and Philouze, 1985), and the traits of *Pto* resistance and fenthion sensitivity cosegregate in genetic crosses (Carland and Staskawicz, 1993; Martin et al., 1993a). The fact that these two traits are separated by less than 0.08 centimorgans (Carland and Staskawicz, 1993) leads to the intriguing possibility that fenthion may be structurally similar to an elicitor produced by *P. s. tomato* strains expressing the *avrPto* avirulence gene. However, in this light it should be noted that although maize plants with the Texas cytoplasm carry a receptor for both the carbamate insecticide methomyl and a toxin produced by *Bipolaris maydis* race T, these molecules bear no obvious structural similarity (Levings, 1990).

The tomato–*P. s. tomato* system is an excellent model for studying the interactions between plants and pathogens because genes from both plant and pathogen that control disease resistance have been cloned. We have taken a genetic approach toward the dissection of the tomato–*P. s. tomato* interaction and have isolated a series of tomato mutants altered in the ability to resist *P. s. tomato* strains expressing *avrPto*. Genetic analysis of these mutants indicated that we have isolated mutations in the *Pto* resistance locus and mutations in a new locus designated *Prf*, for *P*seudomonas *r*esistance and *f*enthion sensitivity. Analysis of these mutants should help to further define this signaling pathway and assist in understanding the molecular events that underlie successful recognition of plant pathogens by resistant hosts.

RESULTS

Isolation of Tomato Mutants Susceptible to *P. s. tomato*

Our approach to understanding the interaction between tomato and *P. s. tomato* was to isolate tomato mutants that had lost resistance to *P. s. tomato* strains expressing *avrPto*. Seed of the *Pto*/*Pto* tomato cultivar Rio Grande-76R (76R; Carland and Staskawicz, 1993) was mutagenized with diepoxybutane (DEB) or fast neutron (FN) irradiation, and M_2 seed was harvested from 2138 plants. M_2 seed families from individual M_1 plants were kept separate to facilitate screening and to ensure that mutants isolated from different M_2 seed lots were independent. Twenty-five M_2 plants from each family were grown to seedling stage and assayed for disease resistance by solution inoculation of *P. s. tomato* T1(*avrPto*), a transconjugant of strain T1 of *P. s. tomato* that expresses a plasmid-borne copy of the *avrPto* avirulence gene recognized by tomato lines carrying *Pto* (Ronald et al., 1992). Although most plants developed no bacterial speck disease symptoms, 11 disease-susceptible individuals were isolated from independent M_2 seed lots and shown to breed true in self-progeny. The majority of existing tomato lines lack the *Pto* resistance locus and are, therefore, susceptible to T1(*avrPto*). This made it important to confirm that the susceptible individuals were derived from 76R. We scored DNA from the susceptible plants for restriction fragment length polymorphisms (RFLPs) specific to 76R; these RFLPs exist due to the introgression of *L. pimpinellifolium* DNA at the *Pto* locus (Pitblado and MacNeill, 1983; Martin et al., 1993a). DNAs from susceptible mutants analyzed with RFLP

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markers linked to the *Pto* locus were found to carry fragments specific for 76R (data not shown), indicating that these lines were true mutants.

Mutants Vary in Degree of Susceptibility to *P. s. tomato*

During the isolation of the mutants, we noticed that individual mutants differed in the severity of the symptoms caused by T1(*avrPto*), suggesting that the mutants differed in the degree to which resistance had been compromised. To quantify the degree to which the mutants were susceptible, the growth of T1(*avrPto*) was monitored in the mutant plants, as described in Figure 1. Bacterial growth in the mutant plants was compared to growth of the same strain in the wild-type resistant line 76R and its near-isogenic–susceptible relative Rio Grande-76S (76S), which differs from 76R in that it lacks the introgressed region containing the *Pto* locus (Pitblado and MacNeill, 1983). Seven of the mutants (*pto-11*, *prf-2*, *prf-3*, *prf-4*, *prf-9*, *prf-16*, and *prf-19*; Figures 1A to 1C) supported growth of T1(*avrPto*) to the same level as 76S and were considered fully susceptible, and four mutants (*pto-1*, *pto-6*, *pto-7*, and *pto-18*; Figure 1A) supported a lower level of growth and were considered to be intermediate susceptible. To determine that the lower level of bacterial growth in the intermediate-susceptible mutants was not due to a reduction in the ability of these mutants to support growth of *P. s. tomato*, we monitored growth of the normally virulent wild-type *P. s. tomato* strain T1 (without *avrPto*) in the mutant plants. T1 grew to an equivalent level (10^6-fold) in 76R, 76S, and all mutant tomato lines (data not shown).

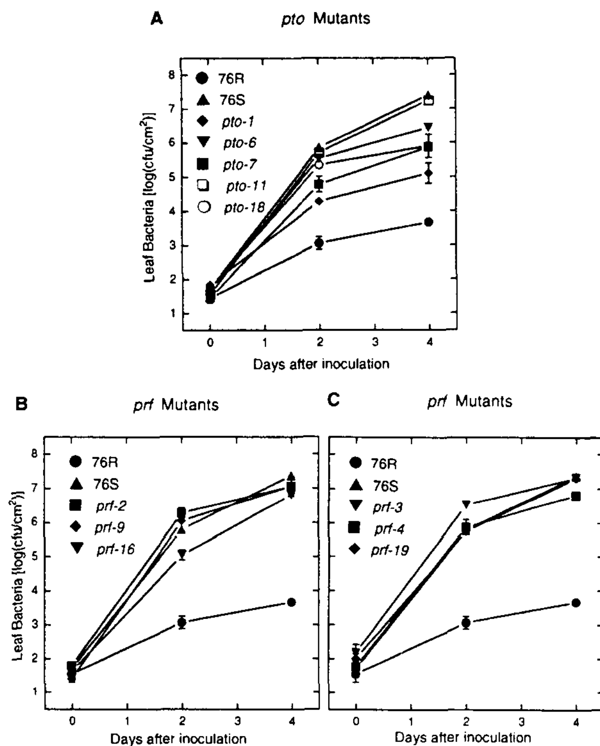

Figure 1. Bacterial Growth in Disease-Susceptible Mutant Tomato Lines.

(A) Growth of *P. s. tomato* T1(*avrPto*) in *pto* mutant lines.

(B) and **(C)** Growth of *P. s. tomato* T1(*avrPto*) in *prf* mutant lines. Tomatoes were inoculated by vacuum infiltration, and bacterial concentrations in plant leaves were assayed after 0, 2, and 4 days. Data points represent the mean of three replicate experiments ± SE.

Mutants Are Specifically Altered in the Ability to Resist *P. s. tomato* Strains

We were interested in finding out if the induced mutations specifically affected the ability of tomato plants to resist bacteria expressing the *avrPto* avirulence gene, or if they affected resistance elicited by other avirulent phytopathogens. To address this question, the mutant plants were inoculated with other bacterial strains to which tomato 76R is resistant. DC3000 is a *P. s. tomato* strain from which *avrPto* was originally cloned, and strain DC3000Δ*avrPto* is a derivative of strain DC3000 in which the genomic copy of *avrPto* was deleted (Ronald et al., 1992). Despite this deletion, DC3000Δ*avrPto* elicited disease resistance specifically on tomato 76R, which suggested the presence of additional avirulence gene(s) in DC3000 for which 76R has resistance specificity (Ronald et al., 1992). DC3000Δ*avrPto* and T1(*avrPto*) were infiltrated into leaves of 76R, 76S, and the mutant plants (see Methods), and disease symptoms were scored after 4 days. The results in Table 1 indicate that all the mutant lines were susceptible to DC3000Δ*avrPto*, with the level of susceptibility of each mutant matching the level of susceptibility to T1(*avrPto*). Identical results were obtained when wild-type DC3000 was used as the inoculum (data not shown). For some of the mutants, susceptibility to DC3000Δ*avrPto* was confirmed by monitoring bacterial growth in infected plant tissue, as described for T1(*avrPto*) in Figure 1. All mutants tested supported levels of growth correlating with the susceptibility levels determined by pipette infiltration (data not shown).

The mutant plants were also inoculated with a strain of the bacterial spot pathogen *Xanthomonas campestris* pv *vesicatoria* expressing the *avrBsP* avirulence gene (Canteros et al., 1991). The *avrBsP* gene elicits disease resistance in all tomato lines tested and is a member of the *avrBs3* gene family, a set of avirulence genes that encodes proteins carrying multiple repeats of a 17–amino acid motif (Bonas et al., 1989). All mutant lines retained the ability to respond with an HR when inoculated with *X. c. vesicatoria* strains expressing the *avrBsP* gene (data not shown). Thus, although the mutants are compromised in the ability to recognize *P. s. tomato* avirulence functions present in DC3000Δ*avrPto* and in *P. s. tomato* strains expressing *avrPto*, they are not altered in a general disease resistance function.

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Table 1. Inoculation Phenotypes of Disease-Susceptible Mutants

		Reaction with Strain at Indicated Inoculum[a]						
		T1		T1(*avrPto*)		DC3000Δ*avrPto*		
Line	Mutagen[b]	5 × 10^5	5 × 10^6	5 × 10^5	5 × 10^6	5 × 10^5	5 × 10^6	Class[c]
76R	—	4.7[d]	5.0	0.0	1.0[e]	0.0	0.9[e]	R
76S	—	4.9	5.0	4.8	5.0	4.8	5.0	S
pto-1	DEB	5.0	5.0	0.6	4.1	1.3	4.2	I
pto-7	DEB	5.0	5.0	1.6[e]	5.0	2.0[e]	4.8	I
pto-18	DEB	4.7	5.0	1.3	5.0	1.7	5.0	I
pto-6	DEB	4.8	5.0	4.6	5.0	4.7	5.0	S
pto-11	DEB	4.8	5.0	4.8	5.0	5.0	5.0	S
prf-2	DEB	4.9	5.0	4.9	5.0	4.9	5.0	S
prf-3	FN	4.8	5.0	5.0	5.0	4.9	5.0	S
prf-4	FN	4.7	5.0	4.8	5.0	5.0	5.0	S
prf-9	DEB	4.7	5.0	4.8	5.0	5.0	5.0	S
prf-16	DEB	4.7	5.0	4.0	5.0	4.1[e]	5.0	S
prf-19	DEB	4.9	5.0	4.6	5.0	5.0	5.0	S

[a] Plants were inoculated by pipette infiltration.
[b] Mutagens used: DEB, diepoxybutane; FN, fast neutron irradiation; (—), not mutagenized.
[c] Class designations: R, resistant, average disease score from inoculations with T1(*avrPto*) or DC3000Δ*avrPto* of 0 to 1 at 5 × 10^5 cfu/mL and 0 to 2 at 5 × 10^6 cfu/mL; I, intermediate, average disease score of 0 to 2 at 5 × 10^5 cfu/mL and 3 to 5 at 5 × 10^6 cfu/mL; S, susceptible, average disease score of 3 to 5 at 5 × 10^5 cfu/mL and 5 at 5 × 10^6 cfu/mL.
[d] Numerical disease scores indicate percentage of the inoculated area in which disease symptoms (characterized by dark necrosis surrounded by chlorosis) were observed: 0 = 0%; 1 = 1 to 10%; 2 = 11 to 40%; 3 = 40 to 70%; 4 = 70 to 99%; 5 = 100%. Standard deviations were less than 1.0. Rankings were derived by averaging results from at least six and in most cases more than 12 inoculations.
[e] SD between 1.0 and 1.5.

Mutations Are Incompletely Dominant and Represent Single Genetic Loci

The induced mutations were determined to be dominant or recessive by crossing each mutant line to 76R and scoring the F_1 progeny for resistance to *P. s. tomato* T1(*avrPto*). As shown in Table 2, the F_1 plants were scored as either resistant or showing intermediate symptoms in the F_1 progeny. Although most of the F_1 progeny were scored as resistant, these progeny consistently showed a slight amount of symptom development (i.e., susceptibility) that was not observed in 76R. In some cases, symptom development was severe enough to warrant classification of the F_1 progeny as intermediate susceptible. Most notably, a large proportion of the F_1 progeny from the 76R × *prf-4* cross displayed were categorized in the intermediate class (Table 2). Together, these results indicated that the mutations are incompletely dominant with respect to the wild-type alleles present in 76R, with the *prf-4* allele showing the highest level of dominance. One or two F_1 plants from each cross that was classified as either resistant or intermediate susceptible was selfed, and the F_2 progeny was scored for resistance to T1(*avrPto*). Segregation of resistance phenotypes in the F_2 was found to be independent of the class designation of the F_1 plant (data not shown), consistent with the different F_1 class designations resulting from incomplete dominance of the mutant alleles. The F_2 segregation ratios observed were consistent with segregation of a single mutation conferring disease susceptibility in each mutant line (Table 2). F_2 progeny from the 76R × *prf-4* cross segregated as resistant, intermediate, and susceptible individuals in a ratio of 1:2:1, consistent with the unique behavior of this allele observed in the F_1 generation.

Mutants Form Two Complementation Groups

One aim of our studies was to identify new components of the tomato resistance signaling pathway responsive to *P. s. tomato* strains expressing *avrPto*. To determine if the mutations we isolated affected the *Pto* locus or other loci required for disease resistance, each mutant plant was crossed to tomato 76S (*pto/pto*). The near-isogenic nature of 76R and 76S implies that 76S should contain all loci necessary for resistance to *P. s. tomato* except for *Pto*. The F_1 progeny of crosses between mutant lines and 76S were scored for resistance to T1(*avrPto*). We reasoned that lines carrying mutations in the *Pto* locus would not be complemented by 76S, whereas lines with mutations in other loci would be complemented and the F_1 progeny would express at least an intermediate level of resistance to T1(*avrPto*). As shown in Table 3, F_1 individuals from crosses of 76S to mutant lines *pto-1*, *pto-6*, *pto-7*, *pto-11*, and *pto-18* exhibited disease development equal to or more severe than that

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observed in the mutant parent. Photographs of the inoculation phenotypes of 76S, *pto-11*, and the 76S × *pto-11* F_1 plant are presented in Figures 2B to 2D, respectively. The wild-type resistant line 76R exhibited no symptom development at this inoculum (Figure 2A). We concluded that these five lines carry mutations in the *pto* locus because the degree of susceptibility of the F_1 progeny demonstrated that the mutant lines were not complemented by 76S. In contrast, F_1 individuals from crosses of 76S to mutant lines *prf-2*, *prf-3*, *prf-4*, *prf-9*, *prf-16*, and *prf-19* showed increased resistance to T1(*avrPto*) compared with the susceptible parents (Table 3). The inoculation phenotypes of 76S, *prf-2*, and the 76S × *prf-2* F_1 plant are shown in Figures 2B, 2E, and 2F, respectively. The resistance expressed in these F_1 plants was comparable to the level of resistance observed for F_1 plants from crosses of 76S to wild-type resistant 76R plants (Table 3). No resistance would have been expected in the 76S × *prf* progeny had the *prf* mutants carried deletions or point mutations at the *Pto* locus. Therefore, we concluded that these six mutants are complemented by 76S and are altered in loci other than *Pto*.

To confirm the ability of *prf-2* to complement 76S, growth of T1(*avrPto*) was monitored in *prf-2* × 76S F_1 plants. Figure 3 shows that there was a 200-fold reduction in the ability of T1(*avrPto*) to grow in the F_1 plant compared with *prf-2* or 76S. The slight increase in growth of T1(*avrPto*) in these plants compared with wild-type resistant 76R can be explained by the incompletely dominant nature of the *Pto* and *Prf* loci (Carland and Staskawicz, 1993; Table 2). To determine if these six *prf* lines represented one or more complementation groups, pairwise crosses between each of *prf-2*, *prf-3*, *prf-4*, *prf-9*, *prf-16*, and *prf-19* were performed, and the resulting F_1 plants were scored for susceptibility to T1(*avrPto*). All F_1 progeny were fully susceptible to the pathogen (data not shown). We concluded that these six lines comprise a single complementation group altered in a novel locus required for pathogen recognition and expression of disease resistance in tomato.

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Table 2. Genetic Analysis of Tomato Mutants

Cross	Class of Mutant Parent[b]	Generation	Number of Plants[a]			χ^{2c}	P
			Resistant[b]	Intermediate	Susceptible		
76R	–	–[d]	10	0	0		
76R × *pto-1*	I	F_1	7[e]	0	0		
		F_2	36	11	0	0.06	0.80
76R × *pto-7*	I	F_1	7	2	0		
		F_2	30	5	0	2.14	0.14
76R × *pto-18*	I	F_1	4	1	0		
		F_2	27	8	0	0.09	0.77
76R × *pto-6*	S	F_1	5	1	0		
		F_2	48	0	18	0.18	0.67
76R × *pto-11*	S	F_1	5	0	0		
		F_2	25	0	6	0.58	0.45
76R × *prf-2*	S	F_1	7	0	0		
		F_2	25	0	7	0.17	0.68
76R × *prf-3*	S	F_1	2	1	0		
		F_2	28	0	5	1.71	0.19
76R × *prf-4*	S	F_1	3	5	0		
		F_2	5	19	11	2.31	0.31
76R × *prf-9*	S	F_1	4	1	0		
		F_2	26	0	9	0.01	0.92
76R × *prf-16*	S	F_1	8	2	0		
		F_2	23	0	11	0.98	0.32
76R × *prf-19*	S	F_1	5	2	0		
		F_2	21	0	11	1.50	0.22

[a] Plants were inoculated by pipette infiltration with *P. s. tomato* T1(*avrPto*) at 5 × 10^5 and 5 × 10^6 cfu/mL.

[b] Class designations are as given in Table 1.

[c] Chi square value was calculated for the expected segregation of the mutation as a single gene ($\chi^2 < 3.84$ is agreeable at $P > 0.05$). Based on the phenotypes of typical F_1 plants, 3:1 segregation of resistant and intermediate plants in the F_2 generation was expected for crosses of 76R to *pto-1*, *pto-7*, and *pto-18*; 1:2:1 segregation of resistant, intermediate, and susceptible plants was expected for the 76R × *prf-4* cross; and 3:1 segregation of resistant and susceptible plants was expected for the other crosses.

[d] The 76R line is not derived from a cross with a susceptible mutant.

[e] Slight disease symptoms were observed in most F_1 progeny of crosses between mutant lines and 76R at the high inoculum concentration, but these were not severe enough to categorize the plant as intermediate susceptible.

Table 3. Segregation of Mutations with the *Pto* Locus

Cross	Generation	Class of Mutant Parent[b]	Number of Plants[a]			χ^{2c}	P
			Resistant[b]	Intermediate	Susceptible		
76S x 76R	F_1	–	3[d]	6	0		
76S x *pto-1*	F_1	I	0	9	0		
	F_2		0	71	25	0.05	0.82
76S x *pto-7*	F_1	I	0	2	3		
	F_2		0	20	48	0.71	0.40
76S x *pto-18*	F_1	I	0	2	4		
	F_2		0	18	40	1.13	0.29
76S x *pto-6*	F_1	S	0	0	3		
	F_2		0	0	61	0.00	1.00
76S x *pto-11*	F_1	S	0	0	6		
	F_2		0	0	65	0.00	1.00
76S x *prf-2*	F_1	S	1	7	0		
	F_2		0	53	41	1.53	0.22
76S x *prf-3*	F_1	S	0	3	0		
	F_2		0	27	32	0.42	0.52
76S x *prf-4*	F_1	S	1	3	0		
	F_2		0	36	34	0.06	0.81
76S x *prf-9*	F_1	S	1	3	0		
	F_2		0	34	30	0.25	0.62
76S x *prf-16*	F_1	S	3	4	0		
	F_2		0	37	27	1.56	0.21
76S x *prf-19*	F_1	S	0	10	0		
	F_2		0	32	30	0.07	0.80

[a] Plants were inoculated by pipette infiltration with T1(*avrPto*).
[b] Class designations are as given in Table 1.
[c] Chi square value was calculated for the expected cosegregation of the induced mutation with the *Pto* locus. Based on the phenotypes of typical F_1 plants, 3:1 segregation of intermediate and susceptible plants in the F_2 generation was expected for the 76S x *pto-1* cross; 1:3 segregation of intermediate and susceptible plants was expected for the 76S x *pto-7* and 76S x *pto-18* crosses; and 1:1 segregation of intermediate and susceptible plants was expected for the crosses of 76S to *prf* mutants.
[d] Slight disease symptoms were observed in most F_1 progeny of crosses between mutant lines and 76S at the high inoculum concentration, but these were not severe enough to categorize the plant as intermediate susceptible.

pto and *prf* Mutants Differ in Response to Fenthion

The mutant lines were employed to address the question of whether the *Pto* locus confers sensitivity to fenthion. A role for *Pto* in fenthion sensitivity has been proposed due to the cosegregation of disease resistance and fenthion sensitivity in genetic crosses (Carland and Staskawicz, 1993; Martin et al., 1993a). Leaflets of mutant plants were treated with fenthion and assayed for the presence of necrotic specks, as displayed by wild-type resistant 76R plants after 4 days. The *pto* mutants were sensitive to fenthion (exemplified by *pto-11* in Figure 2I), displaying necrotic specks similar to those exhibited by wild-type 76R plants (Figure 2G). F_1 progeny from the cross of mutant *pto-11* to 76S were also sensitive (Figure 2J), consistent with the incompletely dominant nature of fenthion sensitivity in tomato (Carland and Staskawicz, 1993). In contrast, mutants in the second complementation group (*prf*) showed no reaction to fenthion (exemplified by *prf-2* in Figure 2K), similar to control 76S plants (Figure 2H). However, F_1 progeny from the cross of mutant *prf-2* to 76S were sensitive (Figure 2L), indicating that *prf-2* was complemented by 76S for fenthion sensitivity as well as resistance to T1(*avrPto*). Together, these results suggested that contrary to previously proposed models the *Pto* locus governs *P. s. tomato* resistance but not fenthion sensitivity. On the other hand, the second complementation group we have identified governs both *P. s. tomato* resistance and fenthion sensitivity, and we have designated this locus *Prf*.

Prf and *Pto* Loci Are Tightly Linked

To determine linkage relationships between the mutations and the *Pto* locus, two F_1 plants from crosses of each mutant line to 76S (genotype *pto Prf/pto Prf*) were selfed, and the F_2 progeny were assayed for resistance to T1(*avrPto*). In the case of each mutant, the F_1 plants selfed had been scored as intermediate susceptible. We expected to find no fully resistant progeny from crosses of *pto* homozygotes to 76S. As shown

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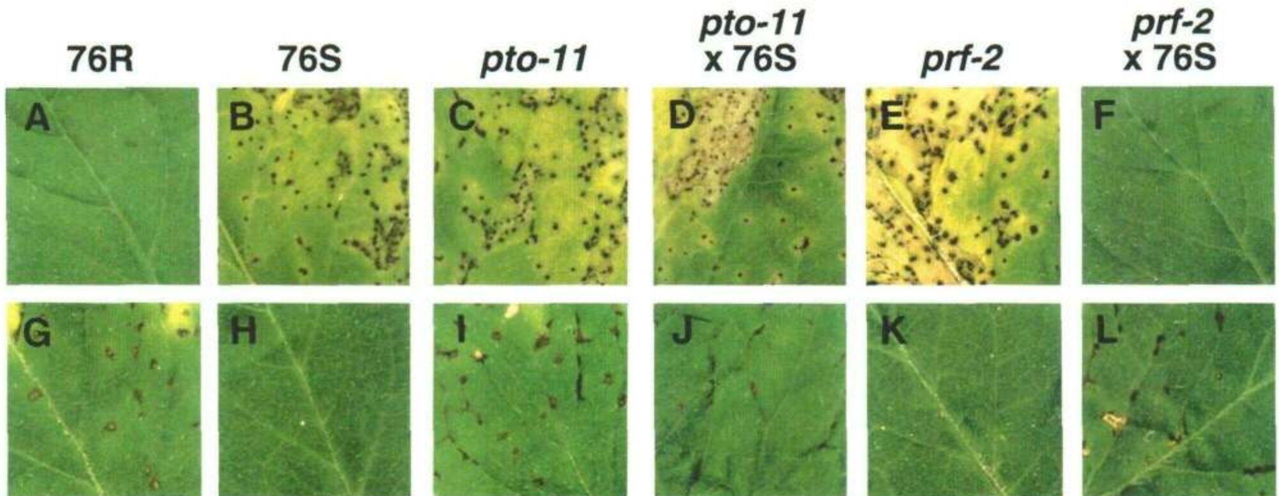

Figure 2. Inoculation and Fenthion Phenotypes of Wild-Type, Mutant, and F_1 Hybrid Tomato Lines.

Tomato lines are indicated at the top. The upper tier of photographs shows the reaction of plants to inoculation with *P. s. tomato* T1(*avrPto*); the bottom tier shows the reaction to fenthion.
(A) 76R inoculated with T1(*avrPto*).
(B) 76S inoculated with T1(*avrPto*).
(C) *pto-11* inoculated with T1(*avrPto*).
(D) F_1(*pto-11* × 76S) inoculated with T1(*avrPto*).
(E) *prf-2* inoculated with T1(*avrPto*).
(F) F_1(*prf-2* × 76S) inoculated with T1(*avrPto*).
(G) 76R treated with fenthion.
(H) 76S treated with fenthion.
(I) *pto-11* treated with fenthion.
(J) F_1(*pto-11* × 76S) treated with fenthion.
(K) *prf-2* treated with fenthion.
(L) F_1(*prf-2* × 76S) treated with fenthion.

in Table 3, this prediction was borne out for all the *pto* homozygotes. F_2 progeny from crosses of the six *prf* homozygotes to 76S were also scored for resistance to T1(*avrPto*). If *Prf* were unlinked to the *Pto* locus, we would expect to recover fully resistant F_2 progeny at a frequency of 1 to 16. Of 415 *prf* × 76S F_2 individuals tested, none was fully resistant to T1(*avrPto*). Chi square analysis of the *prf-2* × 76S F_2 individuals (Table 3) allowed us to reject the hypothesis that *Pto* and *Prf* are unlinked at the 95% confidence limit (χ^2 = 6.67). Instead, fully susceptible and intermediate susceptible F_2 individuals segregated at a ratio of 1:1 (Table 3). These ratios are consistent with the segregation of two tightly linked loci, because these are the phenotypes of the parent and F_1 plants, respectively.

These data indicated that we had not recovered individuals carrying two recombinant alleles (genotype *Pto Prf*/*Pto Prf*) in the F_2 generation. To determine if any of the F_2 individuals were recombinants of genotype *Pto Prf*/*pto Prf* or *Pto Prf*/*Pto prf*, F_2 individuals displaying the highest degree of resistance to T1(*avrPto*) were selfed, and F_3 progeny were assayed for disease resistance. No fully resistant F_3 progeny were identified from any of the populations tested (data not shown). We concluded that none of the F_2 individuals carried a recombinant allele. Together, these data allowed us to conclude that the *Prf* and *Pto* loci are separated by no more than 4.0 centimorgans (χ^2 = 3.87; $P < 0.05$).

DISCUSSION

From our screen for disease-susceptible tomato mutants, we identified lines altered in two loci that play essential roles in the recognition of *P. s. tomato* strains expressing the avirulence gene *avrPto*. Complementation tests using the *pto*/*pto* line 76S demonstrated that five of the lines carry mutations at the *Pto* locus, whereas six additional lines carry mutations at another locus. This locus, designated *Prf* (for *P*seudomonas *r*esistance and *f*enthion sensitivity), is a strong candidate for encoding a second component of the tomato *Pto* disease resistance pathway. It is possible that *Prf* encodes a protein that acts

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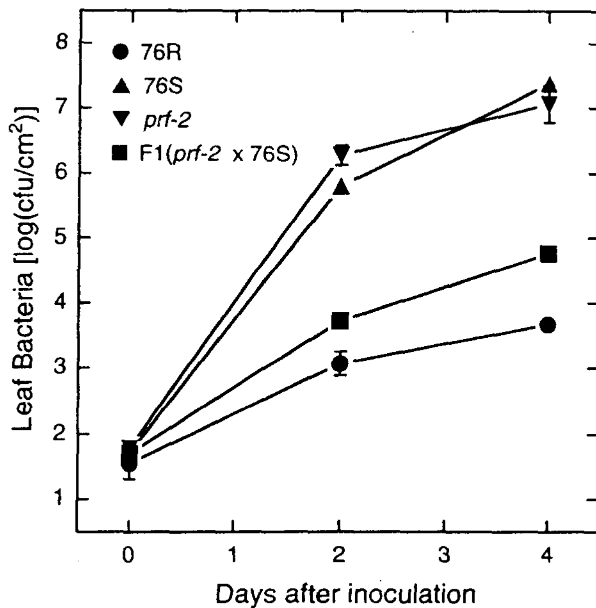

Figure 3. Complementation between Mutant *prf-2* and 76S.

Growth of *P. s. tomato* T1(*avrPto*) was monitored, as described in the legend to Figure 1.

downstream of Pto in signaling that is activated by elicitor(s) expressed by avirulent strains of *P. s. tomato.* Alternatively, *Prf* could encode a receptor for these elicitor(s) or a transcriptional activator of the *Pto* gene. The isolation of the *prf* mutants demonstrated the utility of the mutational approach as a complement to classic breeding for the identification of loci governing disease resistance in that *Prf* is present in both resistant (76R) and susceptible (76S) near-isogenic lines.

We identified five lines that carry mutations in the *Pto* resistance locus. The *pto* mutants vary by 200-fold in the degree to which they restrict growth of *P. s. tomato* strains expressing *avrPto*, with many of the mutants exhibiting phenotypes intermediate between *Pto* and *pto* homozygotes. This variation is interesting and could be due to the effects of different amino acid changes within a single *Pto* gene. Alternatively, these results would be consistent with a model in which more than one member of the *Pto* gene family functions in resistance to *P. s. tomato.* Because there are five to seven homologs of *Pto* at the *Pto* locus (Martin et al., 1993b), mutations in individual homologs could be expected to yield plants retaining the resistance phenotype conferred by the remaining homologs. With the cloning of one member of this gene family (Martin et al., 1993b), it will be possible to address these scenarios through molecular analyses of the *pto* mutant alleles.

In a genetic dissection of barley resistance to *E. graminis*, Jorgensen (1988) isolated 22 mutations in the *Ml-a12* resistance locus, and these also varied widely in the degree to which they affected disease resistance. These results support the concept that plant disease resistance loci are complex (Pryor, 1987). Recently, molecular and genetic analyses of spontaneously generated alterations at the *Rp1* locus of maize, which governs resistance to *Puccinia sorghi*, have suggested that this instability is a result of intralocus recombination (Sudapak et al., 1993). This suggests that *Rp1* may also be comprised of multiple homologous alleles within which unequal exchange may lead to the generation of new resistance specificities (Sudapak et al., 1993).

Previous genetic data have demonstrated that sensitivity to the insecticide fenthion in tomato results from the action of either *Pto* or a closely linked locus (Carland and Staskawicz, 1993; Martin et al., 1993a). Because we find that *pto* mutants retain sensitivity to fenthion, this trait does not appear to be governed by *Pto.* Consistent with this suggestion, it has recently been shown that fenthion sensitivity in tomato can be conferred by a gene distinct from *Pto*, designated *Fen* (G. Martin, personal communication). In contrast, *prf* homozygotes are both pathogen susceptible and insensitive to fenthion. Thus, whatever the function of the *Prf* locus, the effects of mutations in this locus extend to the activities of both the *Pto* and *Fen* genes. With the availability of fenthion and the cloning of the *Pto*, *Fen*, and *avrPto* genes, some of the models concerning the function of *Prf* should be readily testable.

The responses of *pto* and *prf* homozygotes to other avirulent phytopathogens help to define the step at which these loci function in the tomato disease resistance pathway. The *pto* and *prf* mutations block resistance of tomato to strains T1(*avrPto*) and DC3000, which express *avrPto*, and DC3000Δ*avrPto*, which does not. In contrast, *pto* and *prf* mutations have no effect on the resistance of tomato to strains of *X. c. vesicatoria* expressing the *avrBsP* avirulence gene. In the model for tomato disease resistance signaling diagrammed in Figure 4, *Pto* and *Prf* are placed in a pathway distinct from the pathway governing resistance to *X. c. vesicatoria*(*avrBsP*). The different responses of *pto* and *prf* mutants to fenthion suggest that *Prf* functions downstream or upstream of *Pto* at a position common to *P. s. tomato* and fenthion signaling, although further analysis and eventual cloning of the *Prf* locus will be required to ascertain its precise role. Finally, the fact that *pto* and *prf* mutations block resistance to both T1(*avrPto*) and DC3000Δ*avrPto* suggests either that the elicitors produced by these bacterial strains are transduced by the same signaling pathway in tomato or that this pathway branches at a point upstream of the *Pto* and *Prf* genes.

Analysis of F_2 populations from crosses of *prf* and *pto* mutants indicates that the *Prf* and *Pto* loci are tightly linked. Interestingly, in barley the *Ml-a12* locus and a modifier are also linked (Jorgensen, 1988). It is possible that *Prf* resides at the same genetic location as the *Pto* and *Fen* loci with which it is functionally associated. A similar situation operates at the *S* locus controlling self-incompatibility in *Brassica oleracea*, in which two tightly linked genes encoding a glycoprotein and receptor kinase are separated by a maximum distance of 220 kb (Boyes and Nasrallah, 1993). In this regard, it is extremely interesting to note that the protein of known function with highest sequence similarity to *Pto* is the *Brassica S* receptor kinase (Martin et al., 1993b). We have recently identified yeast artificial chromosome clones of the *Pto* region using markers tightly linked to the *Pto* locus (J. Salmeron, C. Rommens,

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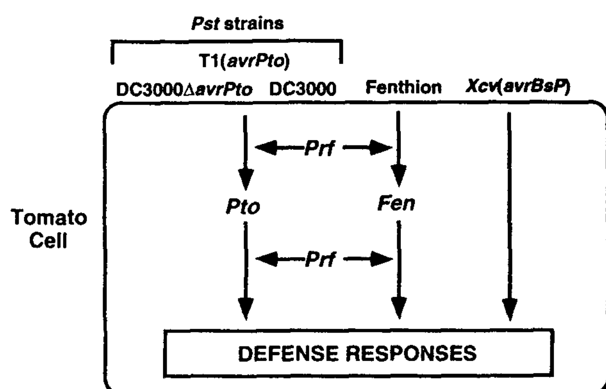

Figure 4. A Model for Genetic Control of Signal Transduction Activated by Avirulent Pathogens and Fenthion in Tomato.

Solid lines represent signaling pathways inferred from genetic analysis of near-isogenic lines differing in the *Pto* locus (Carland and Staskawicz, 1993; Martin et al., 1993a) and mutants altered in pathogen recognition (this study). Evidence for the *Fen* gene comes from the work of G. Martin (personal communication). Two of the possible steps at which the *Prf* locus may act are indicated.

G. Oldroyd, D. Dahlbeck, and B. Staskawicz, unpublished data; Martin et al., 1992), and the linkage between *Pto* and *Prf* should expedite the use of these clones to isolate the *Prf* locus by map-based cloning. The combined approaches of molecular analysis and genetic dissection should lead to a greater understanding of pathogen recognition mediated by the *Pto*/*Prf* pathway and assist the engineering of disease resistance in tomato and other crop plants.

METHODS

Tomato Mutagenesis

To construct tomato (*Lycopersicon esculentum*) lines altered in disease resistance, diepoxybutane (DEB; Emery, 1960) and fast neutron (FN) irradiation (Ververk, 1959; Yu and Yeager, 1960) were employed as mutagenic agents. DEB and FN irradiation are capable of inducing deletions and point mutations in a variety of organisms (Reardon et al., 1987; Sun et al., 1992). To calibrate the level of DEB appropriate for our experiments, seeds of the *Pto*/*Pto* tomato cultivar Rio Grande-76R (76R; Ronald et al., 1992) were imbibed overnight in water and immersed in various concentrations of DEB for 4 hr. The seeds were washed thoroughly with water before planting. The effect of DEB on seed germination was assessed by planting test lots of mutagenized seed. M_1 plants that arose were selfed, and the M_2 populations were scored for segregation of visible mutations. Because doses of 5 to 10 mM DEB resulted in no reduction in seed viability and yielded M_2 families that displayed visible mutations at a frequency of ~5%, these doses were used for large-scale mutagenesis. For FN irradiation, a dose of 15 Gy was demonstrated to be effective and was chosen for our experiments by the International Atomic Energy Agency (Vienna, Austria).

Inoculation Procedures

For mutant screening, M_2 seedlings were grown in the greenhouse for 3 to 4 weeks in 5-inch-diameter clay pots and immersed in a solution of 10 mM $MgCl_2$, 0.05% Silwet L-77 (Union Carbide) containing 2×10^8 colony-forming units (cfu) per mL of *Pseudomonas syringae* pv *tomato* T1(*avrPto*), which carries plasmid pPtE6 bearing the *avrPto* avirulence gene on the wide host range plasmid pDSK519 (Salmeron and Staskawicz, 1993). *P. s. tomato* was grown overnight on agar plates of King's B medium (King et al., 1954) before suspension into 10 mM $MgCl_2$. Susceptible mutants were identified 5 days after inoculation by the presence of necrotic specks surrounded by chlorotic halos, which is symptomatic of bacterial speck disease (Carland and Staskawicz, 1993). At this inoculum level, resistant individuals displayed no visible symptoms. Subsequent inoculations of isolated mutants were performed by pressure infiltration with a plastic Pasteur pipette, using inocula of 5×10^5 and 5×10^6 cfu/mL for *P. s. tomato* strains T1, T1(*avrPto*), DC3000, and DC3000Δ*avrPto* (all described by Ronald et al., 1992).

For testing the ability of mutants to recognize the *avrBsP* avirulence gene, *Xanthomonas campestris* pv *vesicatoria* strain 56 and a transconjugant bearing the *avrBsP* plasmid p965-2 (Canteros et al., 1991; generously provided by G. Minsavage and R. Stall, University of Florida, Gainesville) were infiltrated by pipette at an inoculum of 3×10^8 cfu/mL. Resistance elicited by *X. c. vesicatoria* 56(p965-2) was scored as the appearance of a light grey–brown necrotic patch indicative of the hypersensitive response (HR) 2 days after infiltration. Disease induced by *X. c. vesicatoria* 56 was scored as a dark black–green, water-soaked lesion appearing 3 to 4 days after infiltration. Growth of *P. s. tomato* strains T1, T1(*avrPto*), and DC3000Δ*avrPto* within plants was monitored as described previously (Carland and Staskawicz, 1993).

Fenthion Treatment

Tomato leaflets were immersed in fenthion (Baytex 4; Mobay Chemicals, Kansas City, MO) at concentrations of 0.1 to 1.0 mL/L. Reactions were scored 5 days later as sensitive (displaying black-brown necrotic specks) or insensitive (no reaction) as described previously (Carland and Staskawicz, 1993).

Restriction Fragment Length Polymorphism Analysis

DNA was isolated from mutant plants (Tai and Tanksley, 1990) and digested with restriction endonucleases EcoRV or DraI (New England Biolabs, Beverly, MA). DNA gel blot analysis using the tomato DNA probes CD31A and TG475 (Martin et al., 1993a) was performed as described previously (Carland and Staskawicz, 1993). The same probes were used to confirm that F_1 progenv of crosses of mutant lines to 76S were derived from true crosses.

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