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Tomato *Prf* Is a Member of the Leucine-Rich Repeat Class of Plant Disease Resistance Genes and Lies Embedded within the *Pto* Kinase Gene Cluster

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Summary

In tomato, resistance to Pseudomonas syringae pv. tomato (Pst) strains expressing the avirulence gene *avrPto* requires the presence of at least two host genes, designated *Pto* and *Prf*. Here we report that *Prf* encodes a protein with leucine-zipper, nucleotide-binding, and leucine-rich repeat motifs, as are found in a number of resistance gene products from other plants. *prf* mutant alleles (4) were found to carry alterations within the *Prf* coding sequence. A genomic fragment containing *Prf* complemented a *prf* mutant tomato line both for resistance to Pst strains expressing *avrPto* and for sensitivity to the insecticide Fenthion. *Prf* resides in the middle of the *Pto* gene cluster, 24 kb from the *Pto* gene and 500 bp from the *Fen* gene.

Introduction

Whether plants are resistant or susceptible to attack by a given pathogen is frequently under the control of single dominant resistance genes (Flor, 1971). Resistance gene products are thought to recognize signal molecules produced by the pathogen and to respond by initiating rapid changes in host cell physiology and metabolism that are thought to inhibit pathogen growth directly. The phenotype of this reaction is plant cell necrosis, which is referred to as the hypersensitive response. The hypersensitive response correlates with the generation of activated oxygen species, production of antimicrobial compounds, and reinforcement of host cell walls (Dixon and Lamb, 1990). In addition, responses may include the induction of systemic acquired resistance, which serves effectively to protect the plant against subsequent attack by a broad range of pathogens (Ryals et al., 1995). Disease resistance genes therefore carry dual interest as potential targets for engineering novel resistance specificities into plants as well as for their key role in inducing systemic acquired resistance.

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Resistance gene products are activated in response to pathogen signal molecules termed elicitors. Production of elicitors is controlled by pathogen avirulence genes, of which a large number have been cloned (Long and Staskawicz, 1993; Dangl, 1994). Normally, avirulence and resistance genes are organized in functional pairs such that a given resistance gene is effective only against pathogen strains that express a specific cognate avirulence gene (Flor, 1971; Keen, 1990). However, exceptions to this rule exist. For example, the Arabidopsis *RPM1* gene product (Grant et al., 1995) is involved in the recognition of elicitors produced by Pseudomonas syringae expressing the avirulence genes *avrRpm1* or *avrB* (Bisgrove et al., 1994), suggesting that resistance gene products may function as common points in transduction of distinct pathogen signals.

Recent studies have led to the cloning of a number of plant disease resistance genes (Bent et al., 1994; Grant et al., 1995; Jones et al., 1994; Martin et al., 1993a; Mindrinos et al., 1994; Song et al. 1995; Whitham et al., 1994). A remarkable outcome of this work has been the discovery of similar features among many of these genes, in spite of the diversity of pathogens against which they act. These features include a leucine-rich repeat (LRR), a motif found in a multitude of eukaryotic proteins with roles in signal transduction (Kobe and Deisenhofer, 1994). In addition, sequences predicted to encode nucleotide binding sites and leucine zippers are shared among many resistance genes (Dangl, 1995; Staskawicz et al., 1995). The presence of these motifs and their similar organization among resistance gene products from plants as diverse as tobacco, tomato, rice, flax, and Arabidopsis suggest a common mechanism underlying disease resistance signal transduction throughout the plant kingdom. Two of the major challenges now are to piece together the remainder of the signaling pathways in which these resistance gene products act and to identify the determinants of specificity among proteins recognizing different pathogen species and strains.

A well-studied model for interactions of plant pathogens with their hosts is that between tomato (Lycopersicon esculentum) and P. syringae pv. tomato (Pst; Carland and Staskawicz, 1993; Martin and Tanksley, 1993b). Analyses of naturally occurring resistant and susceptible tomato lines (Pitblado and MacNeill, 1983), as well as mutational studies (Salmeron et al., 1994), have identified two genes required for the tomato signaling pathway that leads to resistance to Pst strains that express the avirulence gene *avrPto* (Ronald et al., 1992; Salmeron and Staskawicz, 1993). One gene, designated *Pto* (Pitblado and MacNeill, 1983), encodes a serine/threonine protein kinase with a potential N-terminal myristoylation site (Martin et al., 1993a) but lacks additional motifs such as a leucine-rich repeat. *Pto* is a member of a tightly clustered family of five genes located on the short arm of chromosome five and encodes a protein highly similar to the cytoplasmic domain of the product of Brassica self-incompatibility gene *SRK* and the mammalian signaling factor *Raf* (Martin et al., 1993a).

The identification of Pto as a protein kinase suggests the importance of intracellular phosphorylation events in the response of tomato to pathogen strains expressing *avrPto*. Indeed, two-hybrid analyses in yeast have recently led to the identification of the tomato Pti1 protein as a substrate for Pto (Zhou et al., 1995). As Pti1 is itself predicted to be a serine/threonine kinase (Zhou et al., 1995), it appears that the pathway for defense against Pst may incorporate a protein kinase cascade similar to those employed in numerous other eukaryotic signaling pathways (Hunter, 1995).

The second gene required for resistance of tomato to Pst, designated *Prf*, was identified through a mutational approach and was shown to be tightly linked to *Pto* (Salmeron et al., 1994). Analysis of *prf* mutant alleles suggests that in addition to its role in disease resistance, the Prf protein also functions in the response of tomato to the organophosphate insecticide Fenthion (Salmeron et al., 1994), a trait which segregates with *Pto* in genetic crosses (Carland and Staskawicz, 1993). In sensitive tomato lines, Fenthion induces rapid necrosis that mimics the hypersensitive response observed after inoculation with Pst strains expressing *avrPto* (Laterrot and Philouze, 1985), suggesting that Fenthion may mimic an elicitor produced under control of the *avrPto* gene in Pst. Necrosis in response to Fenthion does not appear to require the Pto kinase (Martin et al., 1993a; Salmeron et al., 1994) but rather is conferred by another member of the *Pto* gene cluster, designated *Fen*, which encodes a kinase 80% identical in amino acid sequence to Pto (Martin et al., 1994; Rommens et al., 1995). Therefore, Prf is involved with two similar but distinct kinases, Pto and Fen, to induce hypersensitive-like necrosis in response to pathogen elicitor and Fenthion signals, respectively.

Here we report that the *Prf* gene is located within the *Pto* gene cluster. *Prf* encodes a protein with leucine-rich repeat, nucleotide binding, and leucine zipper motifs, which identifies it as a member of the resistance gene class that includes *RPS2*, *RPM1*, *N*, and *L6* (Staskawicz et al. 1995; Dangl, 1995). Significantly, the cloned *Prf* gene complements a tomato *prf* mutant for both disease resistance and Fenthion sensitivity, demonstrating that *Prf*, like Arabidopsis *RPM1*, is a common component for transduction of distinct signals. The finding that Prf contains LRRs demonstrates that at least for the tomato–Pst system, the two major classes of plant disease resistance proteins, LRR–containing proteins and protein kinases, are components of the same signaling pathway.

Results

Construction of YAC and Cosmid Contigs Across the *Prf*/*Pto* Locus

Previously, we had demonstrated tight linkage between the *Prf* and *Pto* genes through analysis of F_2 progeny from crosses of *prf* mutant plants to *pto* mutant lines (Salmeron et al., 1994). Analysis of additional *prf* × *pto* F_2 individuals allowed us to assign *Prf* to a distance of no more than 0.12 cM from *Pto* (see Experimental Procedures). Given the estimated ratio of 220 kb/cM for the region around *Pto*, as derived from analysis of a YAC clone spanning the *Pto* gene (Martin et al., 1993a), we took advantage of the wealth of molecular markers in the vicinity of the *Pto* locus (Martin et al., 1993b) to expedite cloning of the *Prf* gene. Initially, we constructed a contig of 9 YACs in the vicinity of *Pto* by probing available YAC libraries with *Pto*-linked markers (Figure 1B; Martin et al., 1993b). Positioning the ends of these YACs on the genetic map led us to focus on two markers, VC168S and TG538, which mapped to 0.04 and 0.00 cM from the *Pto* locus, respectively (Figure 1A). These markers were used as probes to isolate corresponding cosmids from libraries of 76R (*Pto Prf*/*Pto Prf*) and VFNT Cherry (*pto Prf*/*pto Prf*) DNAs (Experimental Procedures). Cosmid walking from these starting points resulted in the construction of contigs that spanned 80 kb (76R DNA) and 167 kb (VFNT Cherry DNA; data not shown).

Localization of the *Prf* Gene within Cosmid Contigs

Mutations in the *prf* gene (Salmeron et al., 1994) had been isolated with fast neutrons and diepoxybutane, agents that were known to cause deletion mutations in other eukaryotic systems (Reardon et al., 1987; Sun et al., 1992). Given the tight linkage between the *Prf* and *Pto* genes and the availability of cosmids from the *Pto* region, we decided to test for the presence of deletions in *prf* mutant plants that could quickly localize the *Prf* gene. Single-copy probes were identified throughout the cosmid contigs (data not shown) and hybridized to gel blots of *prf* mutant DNAs. A 5.3 kb EcoRI fragment, designated SOR2 (Figure 1C), detected a 1.1 kb alteration in mutant line *prf-3* (Figure 2), a plant isolated by fast neutron bombardment (Salmeron et al., 1994). Fragments adjacent to SOR2 detected no alteration in *prf-3*, suggesting that *prf-3* comprised a simple deletion within the SOR2 fragment. No additional alterations were observed with other probes or in DNAs from other *prf* mutant lines (data not shown).

Complementation of the *prf-3* Mutation by Cosmids Containing SOR2

To test directly whether the region surrounding SOR2 encoded *Prf* activity, cosmids containing SOR2 were introduced into the tomato mutant *prf-3* by Agrobacterium-mediated transformation. Transgenic plants, selected for kanamycin resistance, were inoculated with Pst strain T1 (normally virulent on *Pto Prf* tomatoes) and a transconjugant, T1(*avrPto*), that expressed the *avrPto* avirulence gene and was recognized by tomatoes expressing the *Prf* and *Pto* genes (Ronald et al., 1992). The results shown in Figures 3A–3C and 3H indicate that one SOR2–containing cosmid, pSOR2–7, complemented the *prf-3* mutation, while pSOR1–3 did not complement the *prf-3* mutation (data not shown). As expected, resistance exhibited by the transgenic plants was strictly dependent upon the presence of the *avrPto* gene in the pathogen, as strain T1 caused disease on the pSOR2–7–transformed plants (data not shown). To quantitate the level of resistance conferred by pSOR2–7, we monitored the growth of T1 (*avrPto*) in the transgenic plants. Transgenic plants containing pSOR2–7 displayed a 10000-fold reduction in bacterial growth relative to untransformed *prf-3* plants (Figure 3G). This level

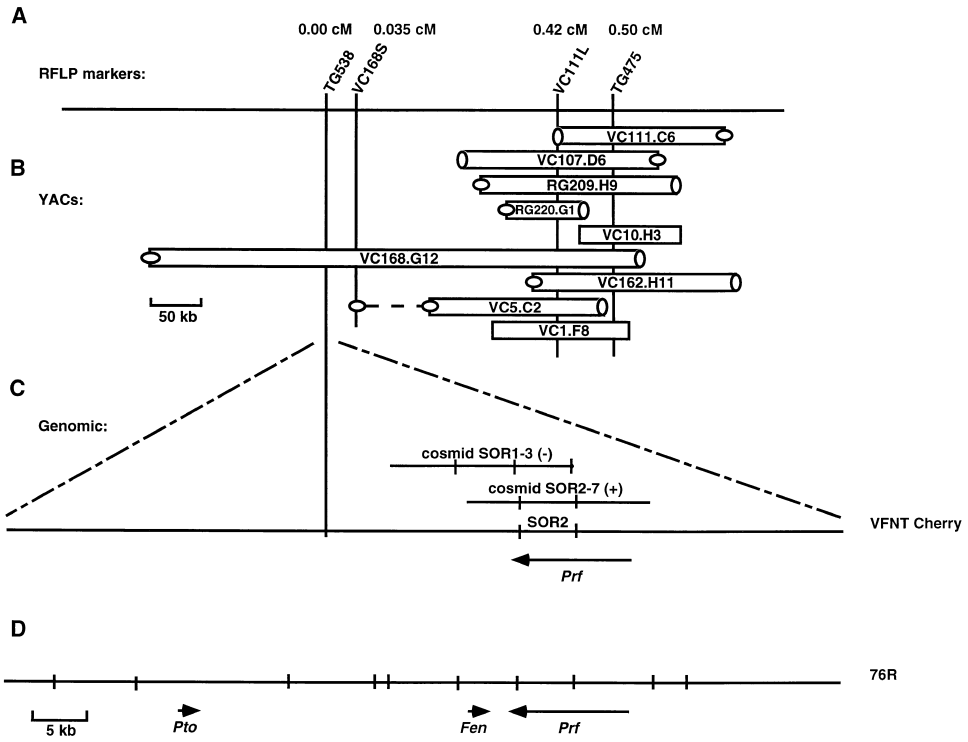


Figure 1. Genetic and Physical Map of the Tomato Genomic Region Containing the *Prf*, *Pto*, and *Fen* Genes

(A) RFLP analysis allowed mapping of molecular markers tightly linked to the *Pto* gene, within a distance to which *Prf* had been genetically mapped relative to *Pto*.

(B) RFLP markers were used to isolate corresponding YACs from available libraries, and a contig of approximately 400 kb was constructed. Identification of a clone (VC168.G12) corresponding to a marker very tightly linked to *Pto* (TG538) motivated subcloning of this YAC into a cosmid contig. The dashed line indicates that marker VC168S was obtained by homology to the left end of YAC VC5.C2.

(C) A cosmid contig of the region containing *Prf* was constructed from VC168.G12. The 5.3 kb EcoRI fragment (SOR2) spanning the 1.1 kb deletion in tomato mutant *prf-3* is indicated. Cosmids (2) from the VC168.G12-derived cosmid contig, SOR1–3 and SOR2–7, were transformed into *prf-3* tomatoes. SOR2–7 complemented the *prf-3* mutation (plus), whereas SOR1–3 did not (minus). Transcript analysis and DNA sequencing defined the boundaries of the *Prf* gene.

(D) The genomic locations of *Pto*, *Fen*, and *Prf* in 76R. The EcoRI restriction map of the contig from RG269.D3 is shown. The location of *Prf* relative to *Pto* and *Fen* was found by PCR and DNA hybridization analysis of the cosmid contig from the YAC RG269.D3 and confirmed by sequencing (see text).

of resistance is comparable to that observed between the wild-type resistant line 76R and the mutant line *prf-3* (Salmeron et al., 1994).

If pSOR2–7 contained the *Prf* gene, then it would also be predicted to confer Fenthion sensitivity to *prf-3* tomatoes. Transgenic lines were treated with Fenthion and reactions scored after 4 days. Whereas *prf-3* tomatoes and pSOR1–3 transformants showed no symptoms following Fenthion treatment, pSOR2–7 transformants developed necrotic specks at least as severe as those observed on wild-type 76R plants (Figures 3D and 3F). These results indicate that pSOR2–7 contains a gene or genes conferring both *Pst(avrPto)* resistance and Fenthion sensitivity in tomato.

To confirm that disease resistance and Fenthion sensitivity in the transgenic plants was conferred by pSOR2–7, pSOR2–7 transformants were test-crossed to *prf-3* mutant plants. Progeny were analyzed for resistance to Pst strains expressing *avrPto* and inheritance of transformed DNA from the vector pCDL04541. A strict correlation between the two traits was observed, indicating that the phenotypes of the transformants were conferred by the introduced cosmid DNA (Figure 3I).

Molecular Cloning of the *Prf* Gene

Complementation of *prf-3* by pSOR2–7 and mapping of the 1 kb deletion in *prf-3* to SOR2 provided strong evidence that the *Prf* coding region lay at least partially within SOR2. To identify genes expressed from the SOR2 region that would be candidates for the *Prf* gene, cDNA libraries constructed from lines 76R and VFNT Cherry were probed with SOR2. Clones of 1.1 and 1.2 kb, respectively, were the longest isolated from each library and were selected as candidate clones for the *Prf* gene.

Analysis of the cDNA clones indicated that the 3′ ends mapped within a 3.8 kb EcoRI fragment downstream of SOR2 and that the clones were partial cDNAs, each containing a single open reading frame extending completely to the 5′ end of the insert. Therefore, we sequenced the entire SOR2 fragment plus 1.05 kb downstream (to a point corresponding to the ends of the cDNA clones) from both 76R and VFNT Cherry DNAs. Primers corresponding to sequences throughout SOR2 were then used to amplify the complete transcribed region of *Prf* from reverse-transcribed 76R mRNA, using both reverse transcription–polymerase chain reaction

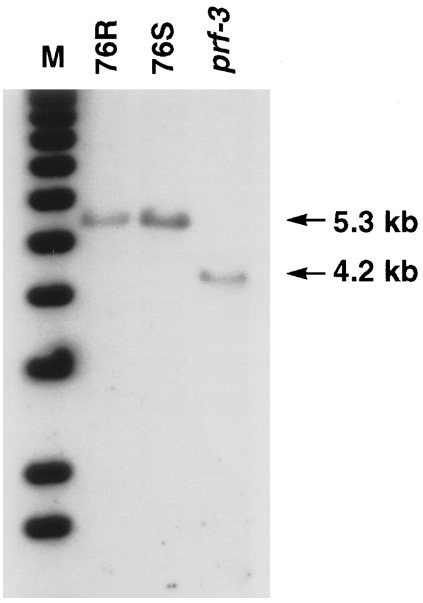


Figure 2. Tomato Mutant *prf-3* Carries a 1.1 kb Deletion

DNAs of the resistant tomato line 76R (*Prf Pto*/*Prf Pto*), susceptible line 76S (*Prf pto*/*Prf pto*), and susceptible line *prf-3* (*prf Pto*/*prf Pto*) were restricted with EcoRI and hybridized to the 5.3 kb SOR2 probe (see Figure 1). M, molecular mass standards.

(RT–PCR) and 5′ rapid amplification of cDNA ends (RACE) approaches (Experimental Procedures). The longest clone obtained from RT–PCR reaction was 4.0 kb in length and was designated pBS-Prf, while the RACE analysis indicated a transcript of 5.7 kb in length. After adding the sequence of the *Prf* 3′ untranslated region as determined from sequencing the shorter cDNA clones (most of this was not incorporated into the RT–PCR reaction products), the full length of the *Prf* mRNA was predicted to be 6.2 kb.

The size of the *Prf* mRNA was confirmed by RNA gel blot analysis. Hybridization to the radiolabeled insert of pBS-Prf revealed an mRNA of approximately 6.2 kb in wild-type 76R leaf tissue (Figure 4, lane 1). This message is approximately 1.1 kb shorter in the *prf-3* mutant (Figure 4, lane 2). Transformants of *prf-3* containing pSOR2–7 express both sizes of mRNA (Figure 4, lane 3). Since RNA for this experiment was taken from uninoculated plant tissue, induction by pathogen attack is not required for expression of the *Prf* gene in tomato.

Nucleotide Sequence of the *Prf* Gene and Analysis of Mutant Alleles

The insert of pBS-Prf, along with the 5′ RACE products, was sequenced and allowed us to predict that the *prf* gene encodes an 1824 amino acid protein of 209.7 kDa (Figure 5B). Analysis of the Prf amino acid sequence shows that the protein falls into the class of resistance gene products recently identified in numerous plant species that contain putative nucleotide binding sites and leucine-rich repeats. Of the three motifs comprising the predicted ATP/GTP binding site, the "P-loop" domain occurs at residues 1120–1132, followed by the companion kinase domains 2 and 3a at 1195–1205 and 1224–1231, respectively. Beginning at residue 1398 is a sequence resembling leucine-rich repeat domains with approximately 14–18 imperfect copies of the leucine-rich repeat motif with a consensus sequence of **LXXLXXLXXLXLXXN/CXXLXXIPSX** (Figure 5C). Other notable features of the Prf protein that are shared by other resistance gene products include a putative leucine zipper, with five complete heptads, which spans residues 959–994. The block of residues from 716–858 comprise two copies of a direct repeat, with 49% amino acid identity between the two copies (Figure 5D). Also present is a string of seven amino acids (1058–1064) that corresponds precisely to one half of the binding site for interleukin-8 in the mammalian interleukin-8 receptor (Hébert et al., 1993).

Analysis of the genomic sequence of *Prf* from 76R revealed the presence of five introns. Two lay within the leader mRNA, including a large 3.6 kb intron occurring 43 nt upstream of the initiator ATG. A third intron occurred between the regions encoding the P-loop and leucine-rich repeat motifs and was located between residues 1436–1437, and an additional two introns occurred in the trailer mRNA (Figure 5A). A comparison of portions of the genomic *Prf* alleles from 76R and VFNT Cherry revealed extremely high similarity, with the encoded proteins 99.2% identical at the amino acid level across the C-terminal 1128 amino acids. This is consistent with genetic evidence showing that naturally occurring lines of tomato that do not carry a functional *Pto*, do carry functional copies of *Prf* (Salmeron et al., 1994).

The mutant alleles from four *prf* plants were amplified from genomic DNAs using *Prf*-specific primers. Partial sequences were determined and compared to the wild-type gene to identify the genetic alterations in the *prf* mutant plants. The *prf-3* mutant was confirmed to carry a simple 1.1 kb deletion between the coding regions for the nucleotide binding site and leucine-rich repeat motifs (see Figure 2), which creates a truncated protein of approximately 1160 amino acids. Two other mutants (*prf-2* and *prf-16*) carried single base changes that resulted in encoded proteins with single amino acid alterations relative to the wild-type sequence. The Prf-2 protein carries a Thr→Ala change at position 1230, which eliminates a residue conserved in the third portion of the nucleotide binding motif, while the Prf-16 protein carries a Tyr→Cys alteration at residue 916. Finally, the *prf-19* allele was found to carry an insertion of a G residue, resulting in a frameshift. The protein encoded by *prf-19* contained a wild-type sequence to amino acid 860, continuing thereafter with Gly and Ser residues before terminating (Figure 5A). These results were based on the nucleotide sequence of the SOR2 region and did not include the entire 5′ end of the gene. It is possible that other mutations also lie within this region. In combination with the complementation data described above, the identification of genetic alterations in four *prf* mutant alleles provides additional evidence that the cDNA we have isolated corresponds to the *Prf* gene.

Homology of *Prf* to Genes in Other Plants

DNA gel blot analysis indicated that a fragment or fragments homologous to *Prf* exist in many plant species

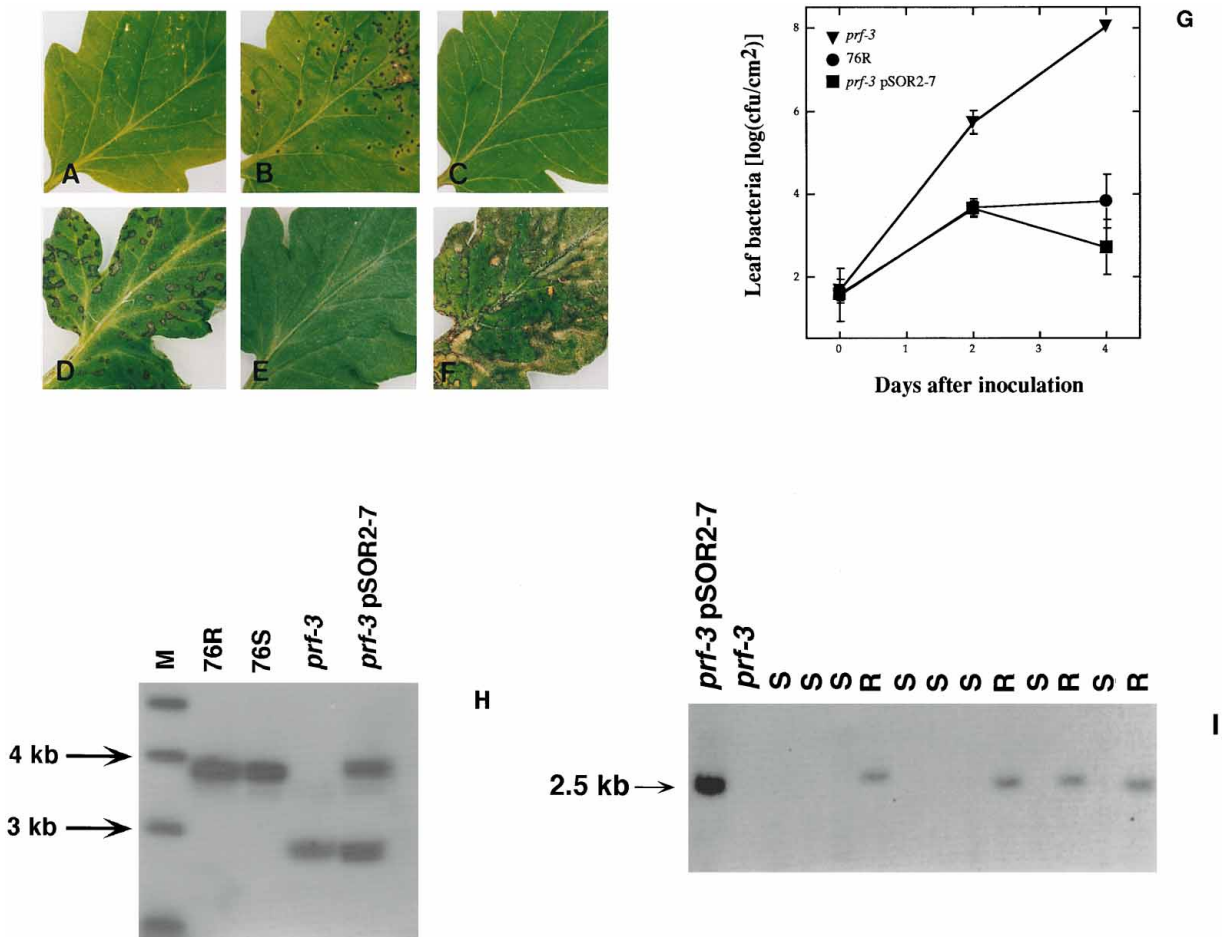


Figure 3. Complementation of the Tomato *prf-3* Mutation by pSOR2–7

(A–F) Reactions of plants transformed with pSOR2–7 to Fenthion and Pst strains expressing *avrPto*.

(A–C) Plants were dipped in a solution of 10 mM $MgCl_2$, 0.05% Silwet L77 (Union Carbide) containing 2×10^8 cfu/ml of Pst strain T1(*avrPto*; Ronald et al., 1992) and photographed after 5 days.

(A) Wild-type 76R inoculated with T1(*avrPto*).

(B) Mutant *prf-3* inoculated with T1(*avrPto*).

(C) Mutant *prf-3* transformed with pSOR2–7 and inoculated with T1(*avrPto*).

(D–F) Plants were dipped in a 4 ml/l solution of Fenthion (Baytex 4; Mobay Chemicals) and photographed after 3 days.

(D) Wild-type 76R treated with Fenthion.

(E) Mutant *prf-3* treated with Fenthion.

(F) Mutant *prf-3* transformed with pSOR2–7 and treated with Fenthion.

(G) Kinetics of bacterial growth in *prf-3* plants transformed with pSOR2–7. Plants were vacuum-infiltrated with T1(*avrPto*) at a concentration of 5×10^4 cfu/ml. Bacterial concentrations in plant leaves were assayed after 0, 2, and 4 days. Data points represent the mean of three replicate experiments plus or minus standard error.

(H) DNA gel blot analysis of the *Prf* locus in the *prf-3* plant transformed with pSOR2–7. Genomic DNA was digested with XbaI, separated on a 0.75% agarose gel, and transferred to a Hybond N membrane. The blot was hybridized with a ^{32}P-labeled probe corresponding to SOR2. Arrows indicate the sizes of molecular mass markers. M, molecular mass standards.

(I) Segregation of resistance to Pst strains expressing *avrPto* and T-DNA in a cross of the *prf-3* mutant lines transformed with pSOR2–7 and *prf-3*. Genomic DNAs were digested with BglII, separated on a 0.75% agarose gel, and transferred to a Hybond N membrane. The blots were hybridized with the 2.5 kb BglII fragment of pCDL04541. Plants were screened for resistance to T1(*avrPto*) by dipping in a solution of 10 mM $MgCl_2$, 0.05% Silwet L-77 (Union Carbide) containing 2×10^8 cfu/ml of T1(*avrPto*) and scored after five days. R, plants resistant to T1(*avrPto*); S, plants susceptible to T1(*avrPto*). The increased intensity of the band in the *prf-3* pSOR2–7 lane is due to the amount of DNA loaded.

(Figure 6). Using moderately high stringency hybridization conditions (see Experimental Procedures), most species tested showed one or two homologous fragments, while a large homologous gene family of approximately nine members was detected in tobacco. Multiple homologous bands were also detected in DNA from resistant tomato plants, indicating that *Prf* is a member of a gene family of approximately eight members.

Positioning *Prf* Relative to the *Pto* and *Fen* Genes

We were interested in determining the physical arrangement of the *Prf*, *Pto*, and *Fen* genes within the *Prf*/*Pto*

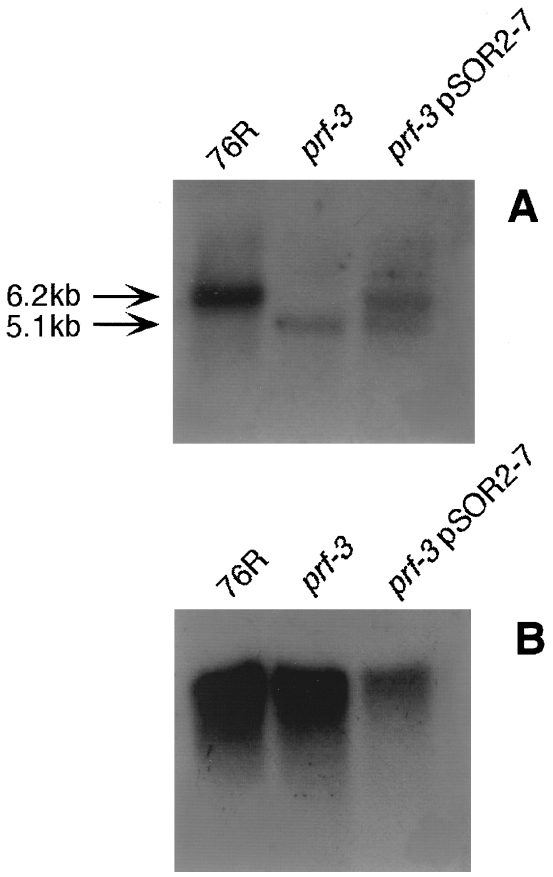


Figure 4. RNA Gel Blot Analysis of *Prf*

Approximately 10 μg of total RNA was separated on a 1.2% agarose gel containing 3.7% formaldehyde and transferred to a Hybond N membrane.

(A) Membrane probed with the insert of pBS-Prf.

(B) Membrane probed with DNA coding for the 18S rRNA.

region. Cosmids containing the *Pto* and *Fen* genes were identified from the 76R contig (see Figure 1D) by PCR amplification of the respective genes from cosmid DNAs, using gene-specific primers. Genes were assigned to individual restriction fragments by probing restricted cosmid DNAs with both the resulting PCR fragments and the cloned *Pto* and *Fen* genes. These data have recently been confirmed by preliminary sequence analysis of the *Prf*/*Pto* region (D. T. L. and R. M. Michelmore, unpublished data). The summary of our results is depicted in Figure 1D. The 3′ end of the *Prf* cDNA is located approximately 500 bp from the open reading frame of the *Fen* gene and approximately 24 kb from the open reading frame of the *Pto* gene.

Discussion

Successful engineering of disease resistance into crop plants will require a thorough understanding of how plants defend themselves from pathogen attack. Recently, progress has been made toward elucidating the genetic control of plant disease resistance on a number of fronts. A major breakthrough came with the cloning of plant disease resistance genes, which demonstrated that diverse plant species utilize proteins with a shared organization of structural motifs for defense against a wide range of pathogens (Staskawicz et al., 1995). These motifs include a "P-loop" region that serves as part of a nucleotide triphosphate binding site and an LRR thought to form a site for interaction with other proteins (Kobe and Deisenhofer, 1994). Existing data indicate that LRR–type plant disease resistance gene products form two subclasses (Jones et al., 1994). Proteins in the first subclass contain the P-loop in the N-terminal half of the protein and the LRR near the C-terminus. In these proteins, the repeats within the LRR tend to be poorly conserved and most closely match the repeat consensus found in yeast adenylate cyclase (Kataoka et al., 1985). These proteins do not contain signal sequences and thus may localize to the plant cytoplasm. The second subclass of resistance gene products lack an apparent nucleotide binding site but contain a signal sequence that may function to target the protein to the cytoplasmic membrane. The repeats within the LRR, found in the N-terminal portion of the protein, are well conserved and most closely resemble those found in plant polygalacturonase inhibitor proteins (Stotz et al., 1994). Prf falls into the first protein subclass. Two other proteins that function in resistance to strains of P. syringae, the Arabidopsis RPS2 and RPM1 proteins (Bent et al., 1994; Grant et al., 1995; Mindrinos et al., 1994), are also members of the first protein subclass, perhaps reflecting a common mechanism by which the elicitors produced under control of the corresponding avirulence genes are presented or perceived.

The fact that proteins that function in resistance to a diverse range of pathogens share a common framework is encouraging for the prospect of engineering novel resistance specificities. However, a much deeper understanding of the mechanisms of resistance will probably be required before such an ultimate goal can be fulfilled.

Figure 5. Molecular Analysis of the *Prf* Gene and Its Product

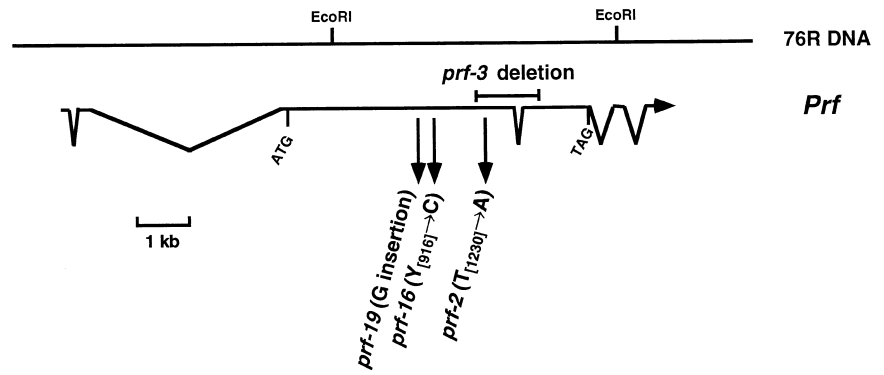
(A) Physical structure of the *Prf* gene, including locations of lesions in *prf* mutations. Top shows an EcoRI restriction map of resistant tomato (76R) DNA in the vicinity of the *Prf* gene. The diagram below represents the *Prf* transcript, with exons indicated by straight horizontal lines and introns indicated by lines angled downward. The initiator (ATG) and termination (TAG) codons are indicated, as is the location of the 1.1 kb deletion in mutant *prf-3*. The locations and amino acid changes of three sequenced mutations are indicated by the downward pointing arrows.

(B) Predicted amino acid sequence of the *Prf* gene product. Residues underlined indicate regions of significance, as described in the text.

(C) Region of internal repetition within the N-terminal half of the Prf protein. Numbers on the left indicate the positions of residues in the Prf amino acid sequence.

(D) Primary structure of the Prf leucine-rich repeat, with consensus listed at bottom. Numbers on the left indicate the positions of residues in the Prf amino acid sequence.

A



B

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IKASKPSRSSSPITVDMVGFIESLLGSVHRALFFISAGPPVSMLDKKLRHLQVFFRLISK   180
RGIEHESMKDLFYHVEDVAYTAAQLCVLGSSCHMDDEFSKFLERISRPFSPGLRQVYLNA   240
LIGLNSSRSKTTMNAKYMLDFVSALQDDLRLRCDNRIRWLQRGLSYLCRFLRDIESYPVS   300
HRQLISLQLNMEDLAIGSANAIYSYDEDMDKTSEIDHELFHLQMKFNYVKVEVDLIRLQN   360
IQGTIIVPMKDLIDYVWEELMFFRSYFMDAFDQFKEQTRITVILNYIQSAVSQAWSVCDS   420
LCHDLNQNDLAREINCLHFQLLLKFKFIKVAIRQMCPSISASSTPDHPMIDLLNFLPMNF   480
EAIDSYSSMLKASCPSSSHRPNRDAESPNTSFLCGPNTDVYSFYSSSSRIPKMDEILKRF   540
HEYILVNLLRKDETNLTFTIADEVKKFYEGLLLMVTYLIEPPVPHTECRKQNDLSMRHEA   600
VAIEAESAVCLHYEDNMNNNSREINQVLQFLTVTFWLIKSEGNLMDLLKHKSTLGNQVLD   660
LIESAHEELILLRSILMDLLRKKLYRLDDLLMHAEVTAKRLAIFSGSCYEYFMNGSSTEK   720
MRPLLSDFLQEIESVKVEFRNVCLQVLDISPFSLTDGEGLVNFLLKNQAKVPNDDAVSSD   780
GSLEDASSTEKMGLPSDFLREIESVEIKEARKLYDQVLDATHCETSKTDGKSFINIMLTQ   840
QDKLPDYDAGSVSYLLNQISVVKDKLLHIGSLLVDIVQYRNMHIELTDLAERVQDKNYIC   900
FFSVKGYIPAWYYTLYLSDVKQLLKFVEAEVKIICLKVPDSSSYSFPKTNGLGYLNCFLG   960
KLEELLRSKLDLIIDLKHQIESVKEGLLCLRSFIDHFSESYDEHDEACGLIARVSVMAYK  1020
AEYVIDSCLAYSHPLWYKVLWISEVLENIKLVNKVVGETCERRNIEVTVHEVAKTTTYVA  1080
PSFSAYTQRANEEMEGFQDTIDELKDKLLGGSPELDVISIVGMPGLGKTTLAKKIYNDPE  1140
VTSRFDVHAQCVVTQLYSWRELLLTILNDVLEPSDRNEKEDGEIADELRRFLLTKRFLIL  1200
IDDVWDYKVWDNLCMCFSDVSNRSRIILTTRLNDVAEYVKCESDPHHLRLFRDDESWTLL  1260
QKEVFQGESCPPELEDVGFEISKSCRGLPLSVVLVAGVLKQKKKTLDSWKVVEQSLSSQR  1320
IGSLEESISIIGFSYKNLPHYLKPCFLYFGGFLQGKDIHVSKMTKLWVAEGFVQANNEKG  1380
QEDTAQGFLDDLIGRNVVMAMEKRPNTKVKTCRIHDLLHKFCMEKAKQEDFLLQINSGEG  1440
VFPERLEEYRLFVHSYQDEIDLWRPSRSNVRSLLFNAIDPDNLLWPRDISFIFESFKLVK  1500
VLDLESFNIGGTFPTEIQYLIQMKYFAAQTDANSIPSSIAKLENLETFVVRGLGGEMILP  1560
CSLLKMVKLRHIHVNDRVSFGLHENMDVLTGNSQLPNLETFSTPRLFYGKDAEKVLRKMP  1620
KLRKLSCIFSGTFGYSRKLKGRCVRFPRLDFLSHLESLKLVSNSYPAKLPHKFNFPSQLR  1680
ELTLSKFRLPWTQISIIAELPNLVILKLLLRAFEGDHWEVKDSEFLELKYLKLDNLKVVQ  1740
WSISDDAFPKLEHLVLTKCKHLEKIPSRFEDAVCLNRVEVNWCNWNVANSAQDIQTMQHE  1800
VIANDSFTVTIQPPDWSKEQPLDS
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C

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716-786 SSTEKMRPLLSDFLQEIESVKV.EFRNVCLQVLDI..SPFSLTDGEGLVNFLLKNQAKVPNDDAVSSDGSLEDA
        |||||| .| ||||.||||||.: | |.:: ||||   :. | |||.:::|::|..|.|:|: ||.| .  |::
787-858 SSTEKM.GLPSDFLREIESVEIKEARKLYDQVLDATHCETSKTDGKSFINIMLTQQDKLPDYDAGSVSYLLNQ.
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D

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1398 VMAMEKRPNTKVKTCRIHDLLHKF
1422 CMEKAKQEDFLLQINSGEGVFPER
1446 LEEYRLFVHSYQDEIDLWRPSRSN
1470 VRSL LFNAIDPDNLLWPRDISFI
1493 FESFKLVKVLDLESFNIGGTFPTE
1517 IQYLIQMKYFAAQTD  ANSIPSS
1539 IAKLENLETFVVRGLGGEMILPCS
1563 LLKMVKLRHIHV
1575 NDRVSFGLHENMDVLTGNSQLPN
1598 LETFSTPRLFYGKDAEKVLRKMPK
1622 LRKLSCIFSGTFGYSRKLKGRCVRFPR
1649 LDFLSHLESLKLVSNSYPAKLPHK
1673 FNFPSQLRELTLSKFRLPWTQISI
1697 IAELPNLVILKLLLRAFEGDHWEVK
1722 DSEFLELKYLKLDNLKVVQWSIS
1745 DDAFPKLEHLVLTKCKHLEKIPSR
1769 FEDAVCLNRVEVNWCNWN VANS
1791 AQDIQTMQHEVIANDSFTVTIQPP

cons LXXLXXLXXLXLXXN/CXXLXXIPSX
```

(*Legend for figure 5 on previous page*)

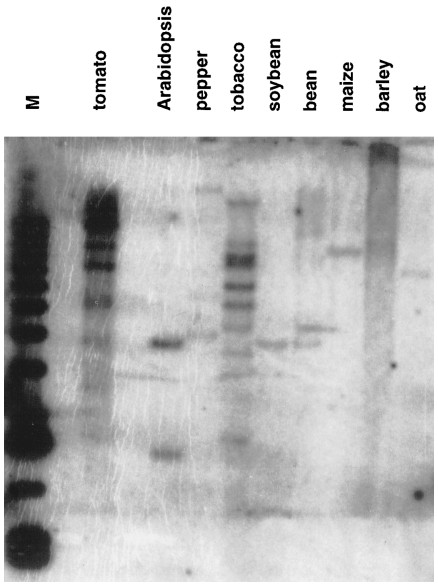

Figure 6. Homologs of the *Prf* Gene Exist in Numerous Plant Species

A fragment encoding most of the Prf leucine-rich repeat was used to probe EcoRI digests of DNAs from the indicated species. For details, see Experimental Procedures.

Fundamental questions such as the cellular localization of resistance gene products and the domains of the protein that provide the specificity of recognition are now potentially answerable. Construction of chimeras between different cloned resistance genes and analysis of naturally occurring and engineered mutant alleles should provide valuable information toward identification of the domains that provide the specificity of recognition. For the *Cf-9* and *Prf* genes, the availability of elicitors or elicitor-like molecules (Fenthion; Carland and Staskawicz, 1993; van Kan et al., 1991) may facilitate these and other studies addressing the roles of resistance gene products in ligand binding and signal transduction.

In tomato, the Pto and Fen kinases are required for transduction of pathogen elicitor and Fenthion signals to induce, in the case of Pto, disease resistance with associated hypersensitivity (Martin et al., 1993a) and in the case of Fen, a hypersensitive-like necrosis (Martin et al., 1994; Rommens et al., 1995). Since *Prf* is required for both these phenotypes (Salmeron et al., 1994), the Prf protein must be a component common in the signaling pathways containing the Pto and Fen kinases. By analogy to some mammalian hormone receptors (Braun et al., 1991) and to the Drosophila Toll protein (Hashimoto et al., 1988), to which the tobacco *N* resistance gene product is similar (Whitham et al. 1994), Prf may function as a receptor that binds either the pathogen elicitor or Fenthion and transduces the signal directly to either of the kinases, which may be membrane-associated. Alternatively, other proteins may serve as intermediaries between Prf and Pto/Fen. It is, of course, equally probable that the Prf protein lies downstream of Pto and Fen in their respective signaling pathways. One component acting downstream of Pto has recently been identified as the Pti1 protein kinase (Zhou et al. 1995). Therefore, if Prf is a downstream component in the tomato resistance pathway, then it is likely that it is the recipient of a signal transduced by one or more protein kinase cascades. The possibility of interactions between each of these kinases and Prf puts forth a number of models that are testable biochemically.

It is curious that among the relatively large number of resistance genes now cloned from a variety of plant species, only one other pathway besides the *Prf*/*Pto* pathway has been demonstrated to involve both an LRR–containing protein and a protein kinase. This is the pathway involved in resistance of rice to bacterial blight, where the *Xa21* gene confers resistance to the causal agent Xanthomonas oryzae (Song, et al., 1995). This example is remarkable in that the LRR and kinase domains both reside on the Xa21 protein (Song, et al., 1995). Possibly, the Prf and Pto proteins are derived from an ancestral tomato resistance factor in which these domains were fused. The physical proximity between the Prf and Pto genes is intriguing and suggests the possibility of such an evolutionary relationship. Are the Prf/Pto and Xa21 pathways unique in their utilization of protein kinases for disease resistance signaling? While this is certainly a possibility at this juncture, it is more likely that the corresponding kinases in other systems have not yet been identified, perhaps due to functional redundancy. Most isolated mutations at the *Pto* locus are weak alleles that cause only partial susceptibility to Pst strains expressing *avrPto*, while mutations at *Prf* completely abolish resistance (Salmeron et al., 1994). This may reflect functional redundancy among different members of the *Pto* gene family in the wild-type plant. Homologs of *Pto* exist in many plant species (Martin et al., 1993a), and if these genes function in disease resistance pathways in their respective hosts, they may also be expected to exist as gene families with multiple functional members.

The *Prf* gene lies embedded within the *Pto* gene cluster, immediately adjacent to the *Fen* gene (Figure 1). The proximity of *Prf* to *Pto* and *Fen*, genes with which *Prf* cooperates in disease resistance signaling, is reminiscent of Brassica species in which two genes that control self-incompatibility, *SLG* and *SRK*, lie within a distance of 200 kb (Boyes and Nasrallah, 1993). By further analogy, the *SRK* gene encodes a receptor kinase proposed to interact with the *SLG*–encoded glycoprotein in initiating the self-incompatibility reaction (Stein et al., 1991), which, like the plant defense response, involves restricting the growth of an invading organism (in this case, the pollen tube).

The potential for Prf to couple with distinct kinases in transduction of different signal molecules may be important in lending the flexibility required by the host to counteract ongoing pathogen evolution. The *avrPto* gene appears to be dispensable for growth of Pst in cell culture and in infected plants (Ronald et al., 1992), and Pst strains lacking *avrPto* are known to arise in fields heavily planted with *Pto* cultivars (T. Suslow, G. E. D. O., and B. J. S., unpublished data). Advantageous for the host would be the ability to recognize altered forms of pathogen elicitors, which may be most easily achieved through differential coupling of distinct but related signaling components. The occurrence of *Pto* and *Prf* as

members of multigene families may allow for additional diversity through recombinational processes (Sudapak et al., 1993) that could prove advantageous to tomato lines in the face of an ever-changing Pst population.

Experimental Procedures

Mapping the *Prf* Gene

To map the *Prf* gene relative to *Pto*, F_2 progeny from crosses of *prf* mutant plants (*prf Pto*/*prf Pto*) to tomato line 76S (*Prf pto*/*Prf pto*; Carland and Staskawicz, 1993) were analyzed for the presence of recombinant chromosomes carrying wild-type alleles of both genes. Out of 413 progeny tested by scoring for resistance to transconjugants of Pst strain T1 containing the *avrPto* plasmid pPtE6 (Ronald et al., 1992), none were recombinant, indicating a maximal genetic distance between *Prf* and *Pto* of 0.12 cM.

Construction of YAC and Cosmid Contigs Spanning the *Prf*/*Pto* Region

All plasmid and cosmid manipulations, preparation of bacterial and yeast media, and hybridization techniques were performed using standard protocols (Ausubel et al., 1992). Tomato RFLP clones TG538 and TG475, which had been previously mapped to the *Prf*/*Pto* region (Martin et al., 1993b), were obtained from Dr. Steven Tanksley (Cornell University). In addition, YAC clones corresponding to TG475, VC111.C6, and VC107.D6 (Martin et al., 1993b), were obtained independently from Drs. Steven Tanksley and Valerie Williamson (University of California, Davis). Ends of these two YACs were isolated and used to identify polymorphic bands between near-isogenic lines 76R (*Prf Pto*/*Prf Pto*) and 76S (*Prf pto*/*Prf pto*), which differ in the presence or absence of the *Pto* gene. The markers could then be mapped relative to *Pto* by probing a mapping population of 1414 F_2 individuals from a cross of 76R to 76S. This revealed that the left end of YAC VC111.C6 was closer to *Pto* than TG475. TG538 and this YAC end were sequenced and oligonucleotides synthesized to create primer pairs corresponding to each marker. Primers for TG538 were $^{5'}$CCAAGTGCAGAGAGTACTGGA$^{3'}$ and $^{5'}$TGAATGAA CATGATCAAAGTATGC$^{3'}$; primers for the left end of YAC VC111.C6 were $^{5'}$ACTCCAGAACCAATGATTGCATA$^{3'}$ and $^{5'}$GGAATTTAAATCTA GAATATCTC$^{3'}$. Primer pairs were used to screen a copy of the Tanksley tomato YAC library obtained from the National Science Foundation Center for Engineering Plants for Resistance Against Pathogens. YAC clones RG209.H9, RG220.G1, VC168.G12, VC162.H11, VC5.2, and VC1.F8 were found to contain the left end of VC111.C6, and clones VC168.G12, RG269.D3, RG669.C9, and RG675.C2 were found to contain TG538. YAC ends were subcloned and mapped relative to other YACs and to the *Pto* gene to construct a contig across the *Prf*/*Pto* region (Figure 1B). Additional markers tightly linked to *Pto* were derived by subcloning fragments from the contig and mapped by probing the 76R $\times$ 76S F_2 population. In this way, RFLP marker VC168S (a copy of the repetitive right end of YAC VC5.C2) was mapped to 0.035 cM from *Pto*, and marker TG538 was mapped to 0.00 cM of *Pto*.

To form cosmid contigs across the *Pto*/*Prf* locus, libraries of 10–20 kb insert size were constructed in pCDL04541 (Jones et al., 1992) from yeast containing either VC168.G12 or RG269.D3. VC168S and TG538 were used as probes to isolate corresponding clones from the cosmid libraries. Cosmid ends were cloned and used in recurrent probing of libraries eventually to form contigs of 167 kb (VC168.G12) spanning VC168S and TG538 and 80 kb (RG269.D3). Cosmids pSOR1–3 and pSOR2–7, from VC168.G12, bear the 5 kb EcoRI fragment SOR2, which contains most of the *Prf* coding sequence.

Cloning the *Prf* Gene

To construct the tomato cDNA library, line 76R was vacuum-infiltrated with a solution of Pst strain T1(*avrPto*) at a concentration of 5×10^7 cfu/ml. Leaf tissue was harvested after 6 hr incubation at room temperature, and the library was constructed using a ZAP-cDNA Synthesis kit (Stratagene). The cDNA library of VFNT Cherry was provided by Dr. Wilhelm Gruissem. Approximately 1.6×10^6 clones were screened from the 76R library, with five hybridizing plaques obtained, and 2×10^5 clones from the VFNT Cherry library, with three hybridizing plaques obtained. The longest cDNA (1.2 kb) was designated *Cdr1*. As an initial step to obtain a full-length cDNA for *Prf*, primers throughout the SOR2 region were used in combination with a primer corresponding to the trailer mRNA of *Cdr1* in PCR reactions, using 76R mRNA as template and a Stratascript kit (Stratagene). The longest clone was obtained using $^{5'}$CCTTCTATTC ATCATCC$^{3'}$ and $^{5'}$CTGCTCCTGATTCTTCT$^{3'}$ as amplification primers. This 4.0 kb band was cloned into the XhoI and XbaI sites of pBluescript-KS(plus) (Stratagene) to form pBS-Prf.

5′ RACE analysis (Frohman et al., 1988) was performed to identify the 5′ end of the *Prf* transcript. The Life Technologies 5′ RACE kit (Catalog Number 18374-025) was used as specified by the manufacturer, except that first-strand cDNAs were tailed with dATP instead of dCTP. The primer T Prime ($^{5'}$TTGCATTGACGTCGACTATCCAGGT TTTTTTTTTTTT$^{3'}$) was substituted for the primer supplied with the kit in all subsequent PCR amplifications. In each RACE experiment, first-strand cDNA was synthesized from 0.25 μg of poly-A^+ RNA isolated from tomato cultivar 76R. Two separate RACE reactions were performed to confirm the 5′ end of the *Prf* transcript. The first experiment used a *Prf*-specific primer PrfPX1 ($^{5'}$TAAGATATGTAACC ATGAGCAACAACCCTTC$^{3'}$) to prime cDNA synthesis. The sequence of PrfPX1 was chosen from analysis of the pBS-Prf insert. After dATP tailing, primers T Prime and PrfPX2 ($^{5'}$GACCTCATCTGCAATAGTA$^{3'}$) were used for PCR amplification. The reaction yielded a 2.0 kb product that was captured in the vector pCRII (Invitrogen). Two clones from this PCR amplification, SS071.7 and SS071.11, were sequenced and indicated transcripts with 5′ ends 5648 nt and 5640 nt, respectively, upstream from the codon terminating the *Prf* open reading frame. The second 5′ RACE experiment was performed using *Prf*-specific primers closer to the 5′ ends mapped by the first RACE reaction. Primer PrfPX1B ($^{5'}$AGGCCCTGCACTGATAAAGAAC AA$^{3'}$) was used to prime cDNA synthesis, and primer PrfPX2B ($^{5'}$AGCA GCTCTGGGATCACTTGCCTT$^{3'}$) was used with T Prime for the PCR amplification. This reaction resulted in a 0.53 kb amplification product that was also cloned in pCRII; five clones were sequenced. The longest two clones (SS074.3 and SS074.12) were homologous to *Prf* and indicated transcripts with 5′ ends 5638 and 5677 bp, respectively, upstream of the termination codon.

DNA Sequencing

The insert of pBS-Prf along with the 5′ RACE products were sequenced either with Sequenase (United States Biochemical Corporation) by the dideoxynucleotide method, or using an Applied Biosystems 373 DNA Sequencer or a Licor DNA sequencer. Sequence data was compiled and analyzed using the Sequencher software (GeneCodes, Inc.). To obtain the sequence of the *Prf* genomic clones, the 5 kb SOR2 fragment was excised from cosmids R207 (from resistant tomato) and pSOR2–7 (susceptible tomato), cut with HindIII, subcloned into pBluescript KS(plus), and sequenced as described above. Subclones of mutant *prf* alleles were amplified from genomic DNAs using Prf-specific primers, ligated into pCRII (Invitrogen), and sequenced.

Complementation

Cosmid pSOR2–7 was introduced into tomato mutant line *prf-3* by Agrobacterium-mediated transformation of excised cotyledons, essentially as described by McCormick et al. (1986). Transgenic plants were identified by resistance to kanamycin (50 μg/ml) and confirmed by DNA gel blot analysis. Transformants were analyzed by inoculation with Pst strain T1(*avrPto*) and exposure to Fenthion, as described previously (Carland and Staskawicz, 1993).

Gel Blot Analysis

DNA and RNA gel blot analysis was performed using standard procedures (Ausubel et al., 1992). In the RNA gel blot analysis, hybridization was performed in the presence of 10% dextran sulfate. For testing for homologs to *Prf* in other plant species, hybridization was performed using a radiolabeled 1.4 kb HindIII fragment from pSOR2–7, corresponding to nucleotides 3150–4494 of *Prf*, under conditions of 65°C, 6 $\times$ SSC. Washing was performed for 1 hr in 0.5 $\times$ SSC, 0.5% SDS, at 65°C. The molecular mass standards used were the 1 kb Ladder (Bethesda Research Laboratories) and the 0.24–9.5 kb RNA Ladder (Gibco BRL).

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