
12. ibid., p. 171.

13. ibid., p. 174.


17. For an example of how field cages can be used to estimate refuges from parasitoid attack, see B. A. Hawkins, F. W. Browning, J. W. Smith Jr., Entomophaga 32, 463 (1987).

18. As an example, our limited data indicate that parasitoids released into field cages that are unable to kill at least 25 to 30% of their hosts (or at least 30% of their hosts sitting on or being fed by both, have a very low probability of being successful, and it would be prudent to continue searches for more efficient species. Quantifying maximum parasitism rates in field conditions could prove valuable for prioritizing potential agents, allowing the best species or combination of species to be released.


43. D. Moore, personal communication.


49. We gratefully acknowledge D. Moore (International Institute of Biological Control, CAB International, Slwood Park), N. J. Mills (Division of Biological Control, University of California, Berkeley), N. Barlow (AgResearch, Lincoln, New Zealand), and W. W. Murdoch (Department of Biological Control, University of Canterbury, Santa Barbara) for providing data or references; M. A. Jervis, N. A. C. Kidd, J. H. Lawton, N. J. Mills, and M. O. Singer for critiques of the manuscript; and M. Rees and M. Holyoak for statistical assistance. This project forms part of the research program of the Natural Environment Research Council Centre for Population Biology and the International Institute of Biological Control.

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Map-Based Cloning of a Protein Kinase Gene Conferring Disease Resistance in Tomato

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The Pto gene in tomato confers resistance to races of Pseudomonas syringae pv. tomato that carry the avirulence gene avrPto. A yeast artificial chromosome clone that spans the Pto region was identified and used to probe a leaf complementary DNA (cDNA) library. A cDNA clone was isolated that represents a gene family, at least six members of which genetically cosegregate with Pto. When susceptible tomato plants were transformed with a cDNA from this family, they were resistant to the pathogen. Analysis of the amino acid sequence revealed similarity to serine-threonine protein kinases, suggesting a role for Pto in a signal transduction pathway.

Gene-for-genotype interactions, in which plant disease resistance involves a single resistance (R) gene in the plant that responds specifically to a single avirulence gene in the pathogen, have been described for numerous plant-pathogen pairs (1). Disease susceptibility results if either the plant R gene or the pathogen avirulence gene is absent from the interacting organisms. Avirulence genes corresponding to specific R genes have been cloned from bacterial and fungal pathogens (2). However, a lack of knowledge about the products of R genes has made their isolation difficult and has hindered efforts to examine their role in the recognition and defense against specific pathogens.

Tomato, Lycopersicon esculentum, offers many advantages for the cloning of R genes solely on the basis of their position on a genetic linkage map. Because tomato has been the subject of more than 50 years of plant breeding, over 27 loci have been identified that confer resistance to many agriculturally important fungi, nematodes, viruses, and bacteria (3). Many of these loci have been located to small intervals on genetic linkage maps (4). A high-density, restriction fragment length polymorphism (RFLP) map (5) and a yeast artificial chromosome (YAC) library have been developed for tomato (6). These resources, combined with the relatively small genome size of tomato (950 megabases) and low level of repeated sequences, have expedited map-
based cloning in this species (7).

We used the interaction of tomato and Pseudomonas syringae pv. tomato, the causative agent of bacterial speck, to isolate a plant R gene by mutagen-based cloning. Resistance to P. syringae pv. tomato in tomato is conferred by the Pto locus on chromosome 5 (8). An avirulence gene (avrPto) from P. syringae pv. tomato induces resistance specifically in tomato cultivars containing Pto (9). Thus, the interaction between tomato and P. syringae pv. tomato involves a gene-for-gene system. With the use of a high-resolution linkage map of the Pto region and closely linked RFLP markers (10), we now have identified a YAC spanning the locus and isolated a cDNA clone of the Pto gene.

The Pto gene was genetically mapped by the scoring of RFLPs and resistance reactions to bacterial inoculation of 251 F₂ progeny derived from a cross between a plant homozygous for the allele (Pto/Pto; Rio Grande–PtoR) and a near isogenic susceptible line [peto/pto; Rio Grande (10)]. One RFLP marker, TG538, cosegregated with the Pto locus (10) and was used to screen a tomato YAC library (6). A 400-kb clone, PTY538-1, was identified that hybridized to this marker (Fig. 1). End-specific probes corresponding to the right (PTY538-1R) and left (PTY538-1L) arms of PTY538-1 were isolated by inverse polymerase chain reaction (PCR) (11) and placed on the high-resolution linkage map of the region (10). The PTY538-1L arm mapped 1.8 centimorgans from Pto, whereas PTY538-1R cosegregated with Pto (Fig. 1). To confirm that PTY538-1 encompassed Pto, it was necessary to identify a plant with a recombination event between PTY538-1R and Pto. We therefore used markers TG538 and PTY538-1R to analyze a total of 1300 plants from various F₂ populations, F₃ families, and over 50 cultivars. One plant was identified that was homozygous for the TG538 allele associated with Pto but was also homozygous for the PTY538-1R allele associated with pto (susceptible allele) (12). All progeny from this plant were resistant to P. syringae pv. tomato, indicating that the plant was homozygous Pto/Pto. This result indicates that PTY538-1 spans the Pto locus.

A sample of DNA from PTY538-1 was isolated from agarose after separation on a clamped homogeneous electric-field gel and used to probe approximately 920,000 plaque-forming units of a leaf cDNA library (13). Of approximately 200 hybridizing plaques, 30 were investigated further. The cDNA inserts were amplified by PCR and used to probe a tomato mapping population consisting of 85 plants with recombination events in the Pto region (10). Two of the clones, CD127 and CD146 (both 1.2 kb), contained sequences that cross-hybridized with each other. When CD127 was mapped, it cosegregated with Pto (Fig. 1). The genetic cosegregation of CD127 with Pto and the fact that the clone was isolated from a leaf-tissue library made the cDNA a strong candidate for the Pto gene.

The CD127 clone detected numerous polymorphic fragments when hybridized with blots of genomic DNA from Rio Grande–PtoR and Rio Grande plants (Fig. 2A). This detection indicated that the clone might contain exons spanning a large region or that it represents a family of related genes. To distinguish between these possibilities, we probed the leaf cDNA library with CD127 insert and isolated an additional 14 cross-hybridizing clones, ranging from 0.6 to 2.4 kb. Analysis of these clones indicated that at least six different expressed genes with homology to CD127 exist in Rio Grande–PtoR (14). To investigate the genome location of the family members, total DNA from the YAC transformant PTY538-1 was digested with Bst NI and analyzed by DNA blot hybridization (Fig. 2B). The YAC contained all of the CD127-hybridizing fragments, with the exception of a 5-kb band that is common to both Rio Grande–PtoR and Rio Grande (Fig. 2B). Therefore, CD127 represents a gene family that is clustered primarily at the Pto locus.

To determine if homologs of the CD127 gene family are present in other plant species, we performed Southern blot analysis on genomic DNA isolated from six dicotyledonous species and five monocotyledonous species. Homologs of CD127 were identified in all species examined (Fig. 2C). Multiple bands were detected in many of the species, indicating the possible presence of a gene family similar to that in tomato. This sequence conservation raises the possibility that other plant species contain genes with structural, and perhaps functional, similarity to the CD127 gene family.

Analysis of RNA blots was used to determine if there were differences in transcript size or abundance produced by the CD127 family members among resistant or susceptible tomato lines (Fig. 3). A prominent 1.3-kb band was observed in resistant and susceptible lines (Fig. 3). A fainter band of 2.5 kb may indicate the presence of less abundant, longer transcripts in the CD127 family. No obvious induction of gene expression was observed upon infection with P. syringae pv. tomato (12).

Genetic complementation tests were performed between tomato and tobacco and between tobacco and potato. The tobacco transformation was performed using the pBI121 plasmid (15). The tobacco transformation resulted in the survival of a clone that cosegregated with Pto and was mapped to the Pto region (16). potato transformation was performed using the pPSu100 plasmid (17). The potato transformation resulted in the survival of a clone that cosegregated with Pto and was mapped to the Pto region (18). These results indicate that the CD127 gene is essential for resistance to Pto in tomato, potato, and tobacco.
conducted to determine if a member of the CD17 gene family confers resistance to P. syringae pv. tomato. Two cDNA clones, representative of the two size classes of transcripts, were initially chosen for this analysis: CD17 (1.2 kb) and CD18 (2.4 kb). The cDNA inserts were placed in the sense orientation into the binary Ti vector pBl121 (Clontech Laboratories, Palo Alto, California) under transcriptional control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The resulting plasmids were designated pPCT5 (CD127) and pPCT8 (CD186). After electroporation of the constructs into Agrobacterium tumefaciens LBA4404, the bacteria were used to transform cotyledon explants of a susceptible tomato cultivar, Moneymaker (15). Transformants containing integrated copies of each construct were identified by kanamycin selection and DNA blot analysis (15).

Five weeks after the transformed plants were transferred to soil, single leaves were inoculated with P. syringae pv. tomato strain T1(pPTE6) carrying avrPto (9, 16). As controls, 4-week-old seedlings of Rio Grande–PtoR, Moneymaker, and Moneymaker transformed with pBl121 alone were also inoculated. Of two plants that were confirmed to contain the integrated transgene from pPCT8, both were resistant to P. syringae pv. tomato strain T1(pPTE6) (17). None of the nine transformants containing integrated copies of pPCT5 exhibited resistant phenotypes (17).

To confirm that P. syringae pv. tomato resistance was due to the introduction of the CD186 cDNA insert, a resistant R0 transgenic plant (PCT8/39) was crossed to a susceptible control plant (cv. Rio Grande). Of 22 backcross progeny examined, nine inherited the CD186 transgene. This result closely fit a 1:1 segregation ratio and indicated that the original integration of pPCT8 sequences in PCT8/39 occurred at a single locus. The same nine backcross-resistant (BC-R) plants containing the CD186 transgene displayed no disease symptoms upon inoculation with T1(pPTE6) (17) (Fig. 4A). The remaining 13 plants lacked the CD186 transgene and displayed typical symptoms of bacterial speck (17) (Fig. 4A). All 22 progeny plants were susceptible to P. syringae pv. tomato strain T1, which lacks avrPto (9, 12).

Because plants showing no disease symptoms after inoculation with T1(pPTE6) might still harbor a large population of P. syringae pv. tomato, we monitored the colony-forming units of bacteria in the progeny and in control plants over a period of 4 days after inoculation (Fig. 4B). The nine BC-R plants exhibiting no disease symptoms contained 105-fold fewer bacteria per square centimeter of leaf area than the backcross susceptible plants at the end of this time period. Lower bacterial populations in BC-R plants than in Rio Grande–PtoR plants were observed and may have been the result of a higher abundance of Pto protein in BC-R plants that resulted from the constitutive 35S promoter. Thus, CD186 functionally complements pto in susceptible plants by inhibiting growth of the P. syringae pv. tomato population and suppressing symptoms of bacterial speck disease.

The entire 2.4-kb DNA sequence of the CD186 insert was determined and a 963-bp open reading frame (ORF1) was found in the region nearest the 35S CaMV promoter in pPCT8 (Fig. 5A). Hereafter referred to as Pto, ORF1 encodes a 321-amino acid hydrophilic protein. The region downstream contains numerous stop codons followed by two regions with homology to Pto. Analysis by PCR confirmed that the structure of the CD186 insert corresponds to the genomic structure in Rio Grande–PtoR. Thus, the CD186 insert may be the result of an infrequent transcriptional read-through event into a pseudogene of the Pto family. Analysis of RNA blots with the entire CD186 insert as a probe detected a prominent 1.3-kb and faint 2.5-kb transcript identical to CD127 (12).

Comparison of the deduced amino acid sequence of Pto with the protein sequences

Fig. 4. (A) Representative plants of (left) the susceptible backcross progeny and (right) the resistant backcross progeny. Plants were inoculated as described with P. syringae pv. tomato strain T1(pPTE6) (16). Photographs were taken 6 days after inoculation. (B) Growth of P. syringae pv. tomato in leaves of tomato. Leaves of 7-week-old plants were inoculated with P. syringae pv. tomato strain T1(pPTE6) and bacterial populations were determined at the specified time points (35). Tomato lines examined were Rio Grande–PtoR (circles), Moneymaker (squares), backcross progeny with (triangles), or without pPCT8 (inverted triangles). Values are the means of three samples, each consisting of three leaf disks from three different plants. Error bars equal one-half of the least significant difference at probability level 0.05. Means are significantly different where error bars do not overlap.

Fig. 5. Physical map of CD186 cDNA and predicted amino acid sequence of Pto. (A) The position and orientation of ORF1 (Pto) and the 3SS CaMV promoter on pPCT8 are indicated. Regions designated a' and b' are 87% and 82% homologous, respectively, to a and b in ORF1. DNA base pairs corresponding to ORF1 a' and b' are shown above the line. Numbers in parentheses refer to positions in ORF1 corresponding to a' and b'. (B) Deduced amino acid sequence of ORF1 (Pto) shown in one-letter code (36). The positions of subdomains characteristic of protein kinases (19) are indicated in parentheses above the sequence. Amino acids that are highly conserved among protein kinases are underlined (19). Residues that indicate serine-threonine specificity are double-underlined (19). A potential myristoylation site (26) at the NH2 terminus is overlined. The nucleotide sequence has been deposited in GenBank (accession number U02271).
contained in GenBank release 77 (18) uncovered similarities with the catalytic domains of many plant, mammalian, and lower eukaryote serine-threonine protein kinases. Eleven subdomains, including 15 invariant amino acids characteristic of protein kinases, are present in the expected locations (Fig. 5B). Sequences indicative of serine-threonine kinase activity occur in subdomains VI (consensus DLXPEN) and VIII [consensus C(T)/S/X/V/F/XAPE (19)]. To examine further its relation to the protein kinases, the Pio sequence was compared to the Protein Kinase Catalytic Domain Database (19). The five most similar matches were to other putative serine-threonine protein kinase genes of plant origin, including three with unknown function from Arabidopsis (TMK1, GenBank accession number L00670; ARK1, number M80238; and RLPK, number M86459 (20)), one of unknown function from maize (ZMPK1, number X52384 (21)), and one from Brassica that is believed to be involved in the recognition of self-pollen by stigma cells (SRK6, number M76647 (22)). The similarity between Pto and SRK6 is particularly interesting because SRK6 appears to be involved in a specific cell-cell interaction (pollen-cell-stigma papillary cell) like that between tomato and P. syringae pv. tomato (22). Other than plants, the closest matches in the database were to mammalian serine-threonine kinases of the Raf family (23).

The observation that transcripts of similar size and abundance are produced from the Pto gene family in both resistant and susceptible tomato lines raises the question of what molecular differences account for the different responses to the pathogen. Two possibilities are that the expression levels differ for specific members of the Pto gene family that confer resistance or that resistant plants contain a gene with a minor sequence difference resulting in a Pto protein of unique activity. Sequence comparisons of DNA among Pto gene family members in resistant and susceptible lines and the development of gene-specific probes and Pio antibodies should help to distinguish between these possibilities.

The presence of short Pio-homologous segments in CD186 in close proximity to Pto raises the possibility that recombination events have occurred in this region, shuffling domains among gene family members. Such intragenic recombination, perhaps by unequal crossing-over, would be increased by the clustering of family members at the Pto locus and could provide a mechanism for generating resistance specific for new races of P. syringae pv. tomato and perhaps to other phytopathogens. Possible occurrence of clustered R gene families in other plant species may explain why several disease-resistance loci (especially those corresponding to different races of the same pathogen) often map within small genetic intervals (24).

The elicitor-receptor model of gene-for-generegion interactions suggests that the products of the R genes function as specific receptors for pathogen elicitors (25). The Pto protein contains no obvious membrane-spanning or extracellular domain that would suggest function as an external receptor for a pathogen signal. A potential myristoylation site occurs at the NH₂ terminus; the protein might therefore be membrane-associated in a manner similar to that of the myristoylated tyrosine protein kinase p60(p21) (26) (Fig. 5B). Association with the plasma membrane could place Pto in proximity to an extracellular receptor having a cytoplasmic domain. Analogous signaling complexes are known in mammals. The elucidation of the Ras pathway suggests that these systems are highly conserved among eukaryotes (27). Alternatively, Pto may exist in the cytoplasmic milieu and participate at a later step of a phosphorylation cascade. By analogy to Ras signaling, it is possible that the activation of Pto leads, by multiple steps, to the activation of defense gene expression. Finally, because there are several members of the Pto gene family in both resistant and susceptible lines and because gene expression does not require P. syringae pv. tomato infection, it seems possible that Pto-like protein kinases may also participate in other cellular activities.

Some elicitors of plant defense responses induce phosphorylation and dephosphorylation of specific plant proteins, and inhibitors of mammalian protein kinases inhibit the expression of certain plant defense genes (28). Thus, kinase-phosphatase activities appeared to be involved in signaling between plants and pathogens. The discovery that a disease-resistance gene encodes a serine-threonine protein kinase establishes a phosphorylation cascade as part of the recognition and response phases of plant defense.

The role of AvrPto protein in eliciting a resistance reaction is unknown. This protein may act by directly or indirectly modulating kinase activity encoded by Pto. In this regard, it is notable that several hypersensitive response and pathogenicity (Hrp) proteins isolated from phytopathogenic Pseudomonas species share homology with proteins from the human pathogen Yersinia that are involved in the secretion of virulence determinants (29). Two of these determinants have serine-threonine protein kinase and phosphatase activities (30). Thus, mechanisms of pathogen recognition in plants and of signal-transduction modulation by bacterial phytopathogens may share features in common with mammalian bacterial pathogenesis.

REFERENCES AND NOTES


13. Six-week-old plants of Rio Granado—Prohib1 and TA20B (Pio1/Pio1) were inoculated by dipping into a solution of avirulent P. syringae pv. tomato strain PT1 (4 x 10⁹ colony forming-units (CFU)/ml), 10 mM MgCl₂, and 0.05% L-77 Silvet (Union Carbide, Southbury, CT) dispersed in distilled water. Leaf tissue was harvested at 2, 6, 22, 48, and 72 hours after inoculation, polyadenylated (poly(A)+) RNA was prepared, and equal amounts of each sample were pooled before cDNA library construction. The cDNA library was constructed in vector agt10 with the use of a mixture of random and oligo(dT) primers (Stratagene).

14. Primers were designed with the use of partial sequence data from both ends of CD127 and used with PCR to amplify a product from the insert of the cDNA clone. PCR products were digested with restriction enzymes recognizing sites with four base pairs (for example, Hae III, Hin II, Taq III), and the fragments were separated in a gel composed of 3% Nuuseae 3TG agarose (FMC Bio-products) and 1% ultrapure agarose. Six different cDNA types were identified on the basis of their patterns of restriction fragments (J. Chunwongse, G. B. Martin, S. D. Tankeley, unpublished results).

15. A. Frany, unpublished results.

16. Leaflets were inoculated as in (13) but with 2 x 10⁶ CFU/ml of the P. syringae pv. tomato strain. Under these conditions, symptoms of bacterial speck appeared after 5 to 7 days on susceptible plants.

17. A resistant reaction was indicated by the absence of necrotic speck on the inoculated leaves. A susceptible reaction was indicated by numerous necrotic speckles species surrounded by chlorotic halos. Reactions were scored 8 to 10 days after inoculation.

18. Comparison was made with the program BLAST, from W. K. Altschul, S. Gish, W. Miller, E. W. Meyers, and D. J. Lipman (J. Mol. Biol. 215, 403 (1990)).

19. The April 1993 version of the data base was used.

Analysis of CD36 Binding Domains: Ligand Specificity Controlled by Dephosphorylation of an Ectodomain

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The protein CD36 is a membrane receptor for thrombospondin (TSP), malaria-infected erythrocytes, and collagen. Three functional sequences were identified within a single disulfide loop of CD36: one that mediates TSP binding (amino acids 87 to 99) and two that support malarial cytoadhesion (amino acids 8 to 21 and 97 to 110). One of these peptides (p87-99) is a consensus protein kinase C (PKC) phosphorylation site. Dephosphorylation of constitutively phosphorylated CD36 in resting platelets and a megakaryocytic cell line led to the loss of collagen adhesion and platelet reactivity to collagen, with a reciprocal increase in TSP binding. PKC-mediated phosphorylation of this ectodomain resulted in a loss of TSP binding and the reciprocal acquisition of collagen binding. In site-directed mutagenesis studies, when the threonine phosphorylation site was changed to alanine, CD36 was expressed in a dephosphorylated state and bound to TSP constitutively.

Regulation of membrane receptor function may be mediated by translocation of the receptor to the cell surface or by association or dissociation of the receptor from regulatory molecules. Although posttranslational modifications such as glycosylation or phosphorylation regulate many protein interactions, the rapid regulation of these characteristics has been confined to events that occur within the membrane or on the cytoplasmic side of the receptor. CD36 is an 88-kDa membrane glycoprotein present on platelets, monocytes, erythroid precursors, endothelial cells, and several tumor cell lines and is a receptor for the adhesive platelet and extracellular matrix protein TSP (1-6), Plasmodium falciparum-infected erythrocytes (7-9), and collagen (10). CD36 is one of several TSP binding sites on platelets, and purified CD36 binds TSP (1-6). In addition, CD36 acts as a receptor that is critical for the cytoadherence of P. falciparum-infected erythrocytes to endothelium (7-9). Both TSP binding and malarial cytoadhesion are inhibited by the same monoclonal antibody (1,11), suggesting that the domains that mediate these events may be related. CD36 is also one of several reported collagen receptors (12).

Random CD36 domains were expressed in a recombinant expression system, and we screened the library by probing with biotinylated purified TSP, a biotinylated peptide (CSVTCCG) (13) corresponding to a CD36 binding domain within TSP (2), and 51Cr-labeled malaria-infected erythrocytes (14). Positively hybridizing clones were subjected to secondary or tertiary rounds of screening and then sequenced. Analysis of the clones identified overlapping consensus sequences as candidate binding domains. Peptides corresponding to the predicted amino acid sequence of these regions were synthesized and examined for TSP binding and malarial cytoadhesion (15).

The domains of CD36 that are responsible for TSP binding and for the cytoadherence of P. falciparum-infected erythrocytes were identified by random domain library screening (RANDOLS) (Fig. 1A) and confirmed by means of synthetic peptides (Fig. 1B). Three functional sequences were identified within a single putative disulfide loop of CD36 defined by Cys1 and Cys241; one that mediates TSP binding (amino acids 87 to 99) and two that support malarial cytoadhesion (amino acids 8 to 21 and 97 to 110). The malarial binding domain, residues 97 to 110 that we have identified, overlaps p93-110 identified as a binding site.