Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene *Pto*

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We have investigated the origin of the *Pto* disease resistance (*R*) gene that was previously identified in the wild tomato species *Lycopersicon pimpinellifolium* and isolated by map-based cloning. *Pto* encodes a serine-threonine protein kinase that specifically recognizes strains of *Pseudomonas syringae* pv. tomato (*Pst*) that express the avirulence gene *avrPto.* We examined an accession of the distantly related wild species *Lycopersicon hirsutum* var. *glabratum* that exhibits *avrPto*-specific resistance to *Pst*. The *Pst* resistance of *L. hirsutum* was introgressed into a susceptible *Lycopersicon esculentum* background to create the near-isogenic line 96T133-3. Resistance to *Pst(avrPto)* in 96T133-3 was inherited as a single dominant locus and cosegregated with a restriction fragment length polymorphism detected by the *Pto* gene. This observation suggested that a member of the *Pto* gene family confers *Pst(avrPto)* resistance in this *L. hirsutum* line. Here we report the cloning and characterization of four members of the *Pto* family from 96T133-3. One gene (*LhirPto*) is 97% identical to *Pto* and encodes a catalytically active protein kinase that elicits a hypersensitive response when coexpressed with *avrPto* in leaves of *Nicotiana benthamiana*. In common with the Pto kinase, the LhirPto protein physically interacts with AvrPto and downstream members of the *Pto* signaling pathway. Our studies indicate that *R* genes of the protein kinase class may not evolve rapidly in response to pathogen pressure and rather that their ability to recognize specific Avr proteins can be highly conserved.

The *Pto* gene in tomato encodes a serine-threonine protein kinase and confers resistance to isolates of *Pseudomonas syringae* that express the avirulence gene *avrPto* (1, 2). The AvrPto protein is believed to be secreted from *Pseudomonas* into the plant cell by a type III secretion system where its interaction with the Pto kinase initiates responses leading to disease resistance (3–6). Only *Pto* alleles that encode proteins which interact with AvrPto in a yeast two-hybrid system can elicit *avrPto*-dependent resistance response (4, 5). Similarly, only AvrPto variants that interact with the Pto kinase possess avirulence activity (7). We have recently demonstrated that the specificity of the Pto–AvrPto interaction is determined by a threonine residue located within the activation loop of the Pto kinase (8), a domain that participates in substrate binding and catalytic regulation in other protein kinases (9).

In nature, it is likely that mechanisms such as horizontal gene transfer and mutation change the types and specificity of *avr* genes present in plant pathogens (10). Consequently it is thought that the host responds by evolving new disease resistance (*R*) genes that recognize new pathogen avirulence specificities. In contrast to *Pto*, the majority of *R* genes cloned to date encode proteins with a region of leucine-rich repeats (LRRs) and a putative nucleotide-binding site (NBS). In these proteins, the LRR domain is hypothesized to mediate recognition specificity through a direct or indirect interaction with the Avr protein (11–14). Recent reports indicate that the sequences of some LRR-type *R* genes are particularly subject to rapid evolution (15–17) and that regions of the LRR proteins implicated in recognition specificity are affected by diversifying selection (16, 17). Furthermore, the organization of many *R* genes in clustered families appears to make them amenable to unequal crossing over and gene conversion events. These rearrangements give rise to duplications and deletions of LRR regions and to recombination between *R* genes, all of which can alter recognition specificities (16–19).

Despite the apparent ability to evolve rapidly, recent data suggest that some recognition specificities are ancient and that hosts and pathogens may have coexisted for millions of years. One example of an ancient *R* gene specificity occurs in *Arabidopsis,* where a homolog of the *RPM1* gene was cloned from an isolate of *Arabidopsis lyrata* that is resistant to *Pseudomonas syringae*(*avrRpm1*) (20). Although it was not confirmed that the *A. lyrata RPM1* gene actually conferred *Pseudomonas* resistance, this report suggests that recognition of *avrRpm1* predates the divergence of *Arabidopsis thaliana* from *A. lyrata* (20). In fact, *avrRpm1* recognition may have arisen even earlier, as *avrRpm1* (and *avrB)* recognition also has been reported in soybean (21).

We reported previously that an accession of *Lycopersicon hirsutum* var. *glabratum*, PI134418, is resistant to strains of *P. syringae* pv. tomato (*Pst*) that express *avrPto* but is susceptible to strains that do not express this avirulence gene (22). Both restriction fragment length polymorphisms (RFLPs) and morphological features indicate that *L. hirsutum* is a distant relative of *Lycopersicon pimpinellifolium,* from which the *Pto* gene was originally isolated (23). In earlier work the *Pst*(*avrPto*) resistance of PI134418 was introgressed into a susceptible *Lycopersicon esculentum* variety to create the near-isogenic line 96T133-3 (22). In progeny derived from a cross with this line *Pst*(*avrPto*) resistance cosegregates with an RFLP detected by the *Pto* resistance gene and maps to the same location on chromosome 5 as the *Pto* gene in *L. pimpinellifolium* (22). These observations raised the possibility that a member of the *Pto* gene family in *L. hirsutum* is responsible for conferring resistance to *Pst*(*avrPto*).

We now report the cloning and molecular characterization of four *Pto* gene family members from accession PI134418 and demonstrate that one of them encodes a protein conferring *avrPto*-specific disease resistance. Our results indicate that the evolution of *Pto* recognition specificity for the *Pseudomonas* AvrPto protein predates the divergence of *L. pimpinellifolium* and *L. hirsutum*.

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Abbreviations: LRR, leucine-rich repeat; RFLP, restriction fragment length polymorphism; *Pst, Pseudomonas syringae* pv. tomato; GST, glutathione *S*-transferase; HR, hypersensitive response.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF318490, AF318491, AF318492, and AF318493 for *LhirPtoE, LhirPtoF, LhirPtoB,* and *LhirPtoD*, respectively).

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Experimental Procedures

Standard Methods and Plant Treatments. Standard methods were used for tomato genomic DNA isolation, restriction enzyme digestion, DNA blotting, and DNA gel blot hybridization (24). Bacterial growth measurements were performed as described previously (1). Fenthion sensitivity assays were performed by vacuum infiltrating 4-week-old tomato plants with a solution containing 0.05% fenthion (Chem Service, West Chester, PA), 0.15% isopropyl alcohol, and 0.004% Silwet (OSI Specialties, South Charleston, WV) for 2 min.

Plant Materials. Seeds of 96T133-3, TA537, and TA209 were a gift of Steven Tanksley (Cornell Univ.). Line 96T133-3 was developed by introgressing *Pst* (*avrPto*) resistance into cultivar TA209 as described in ref. 22. TA537 was developed by a similar process, which introgressed the *Pto* locus from an Israeli processing line, H14-Pto, into TA209 (25).

cDNA Library Construction and cDNA Cloning. mRNA was isolated from 96T133-3 leaves 6 h after infiltration with 10^7 colony-forming units/ml *Pst* (*avrPto*) and used to construct a cDNA library in a ZAP Express cDNA kit (Stratagene). Plaque lifts were performed on $>10^6$ plaques according to the Stratagene protocol. Filters were hybridized with radiolabeled *Pto* probe and washed to a stringency of 0.5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). MEGALIGN 4.02, part of the DNASTAR package (DNASTAR, Madison, WI) was used to create sequence alignments and dendrograms.

Yeast Two-Hybrid Analysis. Primers which introduced a *Bam*HI or a *Sal*I site to the 5′ and 3′ ends, respectively, were designed for the *LhirPto* open reading frames. The LhirPto inserts were PCR amplified by using *Pwo* high-fidelity polymerase (Boehringer Mannheim), cloned into pBluescript (Stratagene) and confirmed by sequencing. *LhirPto* genes were cloned into the pEG202 bait vector and transformed into yeast strain EGY48 by using the lithium acetate method (24). Western blots and two-hybrid assays were done as previously described (26–28).

Glutathione *S*-Transferase (GST) Fusion Protein Expression and Kinase Assays. Primers that introduced a *Bam*HI or a *Sal*I site to the 5′ and 3′ ends, respectively, were designed for each *LhirPto* open reading frame. The cDNA insert was PCR amplified by using *Pwo* high-fidelity DNA polymerase (Boehringer Mannheim), cloned into pBluescript (Stratagene), and confirmed by sequencing. The insert was removed from pBluescript, ligated into pGEX-KG, and electroporated into *Escherichia coli* strain DH5α. GST-fusion protein purification and phosphorylation assays were performed as previously described (27, 29, 30). Autoradiography was performed with a Storm System Imager (Molecular Dynamics). Purified Pti1 and Pti4 were gifts from Yong Gu (Boyce Thompson Institute).

Transient Assays. *LhirPto* genes were removed from pBluescript (see above), ligated onto the cauliflower mosaic virus 35S promoter, and cloned into pBTEX or pBTEX::*35S*::*avrPto*. These constructs were electroporated into *Agrobacterium tumefaciens* strain EHA105. Transient assays were performed as described previously (8).

Results

Tomato Line 96T133-3 Contains the *Pto* Locus from *L. hirsutum* Accession PI134418. In common with *L. pimpinellifolium,* the original source of the *Pto* resistance gene, *L. hirsutum* var. *glabratum* (accession PI134418) is resistant to strains of *Pst* that express the avirulence gene *avrPto* and is susceptible to strains of *Pst* that do not express *avrPto* (22). Hybridization of a genomic DNA blot with the *Pto* gene revealed that PI134418 contains a *Pto* gene family that is polymorphic with respect to either Rio Grande-PtoR (RG-PtoR) or Rio Grande-PtoS (RG-PtoS) (Fig. 1). These near isogenic lines contain the *Pto* locus from *L. pimpinellifolium* and *L. esculentum*, respectively. Resistance to *Pst*(*avrPto*) was introgressed from PI134418 into the susceptible *L. esculentum* cultivar TA209 (22). The introgression involved six backcrosses to the *L. esculentum* parent with selection in each generation for *avrPto*-mediated resistance, and a final selfing to create the homozygous resistant line 96T133-3. All RFLPs detected by *Pto* in PI134418 were also detected in 96T133-3 (Fig. 1) and cosegregated with *Pst(avrPto)* resistance. Furthermore, the *Pto* RFLP and *Pst*(*avrPto*) resistance mapped to the same location on chromosome 5 as the *Pto* gene found in *L. pimpinellifolium* (22). These data suggested that a member of the clustered *Pto* gene family in this *L. hirsutum* line is responsible for conferring resistance to *Pst*(*avrPto*).

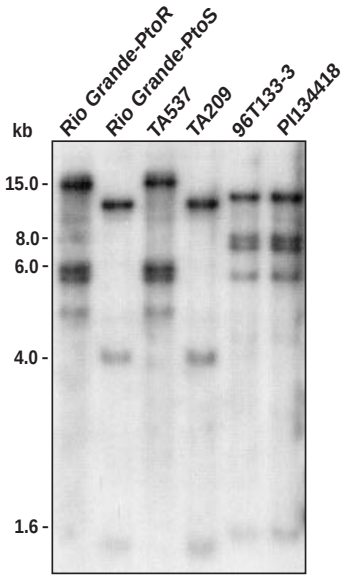

Fig. 1. RFLP patterns detected by the *Pto* gene in wild *L. hirsutum*, *L. esculentum*, and near-isogenic *L. esculentum* lines. Genomic DNA (3 μg) isolated from each of the indicated lines was digested with *Eco*RI, separated on a 1% agarose gel, and transferred onto Hybond N+ membrane. The membrane was hybridized to a ^{32}P-labeled PCR product of the *Pto* open reading frame and washed to a stringency of 0.5× SSC at 65°C. Rio Grande-PtoR and TA537 contain the *Pto* locus from *L. pimpinellifolium*, Rio Grande-PtoS and TA209 contain the *Pto* locus from *L. esculentum*, and 96T133-3 contains the *Pto* locus from the *L. hirsutum* tomato accession PI134418.

The *L. hirsutum* and *L. pimpinellifolium Pto* Loci Confer Identical Levels of Resistance to *P. syringae*. To compare the resistance conferred by the *L. hirsutum* and *L. pimpinellifolium Pto* loci, we studied their abilities to suppress the growth of *Pst* in near-isogenic tomato lines. The *L. pimpinellifolium Pto* locus was introgressed into the susceptible TA209 *L. esculentum* background to create the near-isogenic line TA537 (Fig. 1). Five days after inoculation, *Pst(avrPto)* populations in leaves of 96T133-3 were equivalent to those in TA537 and were 10^4 times lower than in susceptible TA209 leaves (Fig. 2*A*). Neither 96T133-3 nor TA537 showed disease symptoms 1 week after being inoculated with *Pst(avrPto).* However, TA209 developed bacterial speck symptoms 3 days after inoculation. All three lines were susceptible to *Pst* lacking the *avrPto* gene (Fig. 2*B*). These data demonstrate that the *L. hirsutum Pto* locus confers a level of *avrPto*-specific resistance indistinguishable from that conferred by the *L. pimpinellifolium Pto* locus.

The *L. hirsutum Pto* Locus Does Not Confer Fenthion Sensitivity. In *L. pimpinellifolium Pto* is closely linked to the *Fen* gene, which confers sensitivity to the insecticide fenthion (31). Given that the *LhirPto*

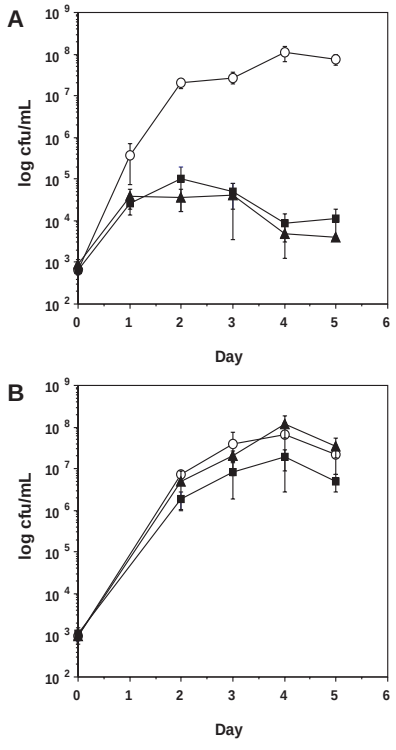

Fig. 2. Growth of *Pst*(*avrPto*) (*A*) or *Pst* (*B*) in leaves of near-isogenic lines containing different *Pto* loci. Four-week-old plants of TA209 (*L. esculentum* *Pto* locus, ○), TA537 (*L. pimpinellifolium* *Pto* locus, ■), and 96T133-3 (*L. hirsutum* *Pto* locus, ▲) were vacuum-infiltrated with 10^5 colony-forming units per milliliter (cfu/ml) of *Pseudomonas,* and bacterial growth was measured as cfu/ml at the time points specified. Each time point represents the average of three samples. Each sample contains three 1-cm^2 leaf discs in 1 ml of 10 mM $MgCl_2$. The error bars represent the standard error.

locus confers *avrPto*-specific resistance, we tested whether 96T133-3 might also exhibit fenthion sensitivity. TA209, TA537, and 96T133-3 leaves were infiltrated with a solution of 0.05% fenthion and observed over a 4-day period. Only TA537, which contains the *Pto* locus from *L. pimpinellifolium,* developed necrotic specks typical of the fenthion response (data not shown). Neither TA209 nor 96T133-3 exhibited symptoms of fenthion sensitivity, indicating that, like *L. esculentum*, 96T133-3 does not contain a functional *Fen* gene. Previous tests for fenthion sensitivity of PI134418 and other *L. hirsutum* accessions have yielded similar results (22, 32).

96T133-3 Expresses Genes That Are Orthologous with *Pto* Gene Family Members from Rio Grande-PtoR and VFNT Cherry. The linkage analysis described above suggested that a member of the *Pto* gene family in 96T133-3 is responsible for conferring resistance to *Pst*(*avrPto*). To isolate members of the *Pto* gene family from 96T133-3, we hybridized a 96T133-3 cDNA library with the *Pto* gene. Restriction fragment analysis of 46 independent cDNAs indicated that there were four different classes of cDNAs (data not shown). We sequenced representatives from each class and found that each class represented a different *LhirPto* gene that shared between 83% and 97% nucleotide identity with *Pto*. Each gene contained an open reading frame encoding a protein that is 314–323 amino acids in length (Fig. 5, which is published as supplemental data on the PNAS web site, www.pnas.org).

The entire *Pto* locus has been sequenced from the tomato lines Rio Grande-PtoR (RG-PtoR) and VFNT Cherry (VFNT) which are resistant and susceptible to *Pst*(*avrPto*), respectively (GenBank accession nos. AF220602 and AF220603). An analysis of these data indicated that the RG-PtoR and VFNT genes present at corresponding locations within the *Pto* gene cluster (orthologs) are more similar to each other than they are to other genes within their respective haplotypes (paralogs) (18). On the basis of these data the *Pto* family members were named by assigning each gene a prefix to denote the species from which it was identified, and a suffix that arbitrarily numbers each family member. Orthologs in VFNT and RG-PtoR were given the same number but were not numbered in any particular order (GenBank accession nos. AF220602 and AF220603). To simplify future comparisons of the *Pto* loci from various tomato species we propose a new nomenclature for the *Pto* locus. With this system a prefix will identify the wild species from which the gene was isolated, and a letter suffix will denote the location of the gene along tomato chromosome 5, based on its orthology to the RG-PtoR *Pto* genes. A summary of our proposed nomenclature is presented in Table 1.

We compared the nucleotide sequences of the *LhirPto* genes with the *Pto* gene families from RG-PtoR (*LpimPto*) and VFNT Cherry (*LescPto*). A dendrogram based on nucleotide identity placed each *LhirPto* gene with the orthologous genes from RG-PtoR and VFNT (Fig. 3). We named each *L. hirsutum Pto* gene according to its putative ortholog from RG-PtoR and VFNT by using the nomenclature presented in Table 1. *LhirPtoE* is most closely related to the *Pto* gene from *L. pimpinellifolium* (RG-PtoR), with 97% nucleotide identity. Because of its close sequence identity (and functional identity, see below) with *Pto* we refer to *LhirPtoE* as simply *LhirPto*. Interestingly, the *Pto* and *LhirPto* genes do not appear to have an ortholog in the susceptible VFNT haplotype (GenBank accession no. AF220603). *LhirPtoB* is most closely related to the genes *Fen* (*LpimPtoB*) and *fen* (*LescPtoB*) and shares 95% and 96% nucleotide identity with them, respectively (26, 31). Because of its close sequence identify to *Fen* and *fen* we refer to *LhirPtoB* as *Lhirfen.* *LhirPtoD* is nearly identical to the genes *LescPtoD* (99% nucleotide identity) and *LpimPtoD* (98% nucleotide identity) genes from VFNT and RG-PtoR haplotypes, respectively. *LhirPtoF* is 93%

Table 1. Proposed nomenclature for the *Pto* gene family

Rio Grande-PtoR ortholog		VFNT ortholog		*L. hirsutum* ortholog
Current*	Proposed†	Current*	Proposed†	Proposed†
*LpimPth4**	*LpimPtoA*	*LescPth4*	*LescPtoA*	—
Fen	*LpimPtoB/Fen*	*fen*	*LescPtoB/fen*	*LhirPtoB/Lhirfen*
LpimPth3	*LpimPtoC*	*LescPth3*	*LescPtoC*	—
LpimPth2	*LpimPtoD*	*LescPth2*	*LescPtoD*	*LhirPtoD*
Pto	*LpimPtoE/Pto*	Not present	Not present	*LhirPtoE/LhirPto*
LpimPth5	*LpimPtoF*	*LescPth5*	*LescPtoF*	*LhirPtoF*

*Nomenclature for *Pto* orthologs and paralogs is based on genomic sequences of the *Pto* loci from Rio Grande-PtoR and VFNT Cherry (GenBank accession nos. AF220602 and AF220603, respectively).

†Proposed gene names are based on their species of origin and designated in alphabetical order according to their position within the *Pto* loci of *L. pimpinellifolium* and *L. esculentum.* A — denotes that cDNAs corresponding to these orthologs were not found in this study.

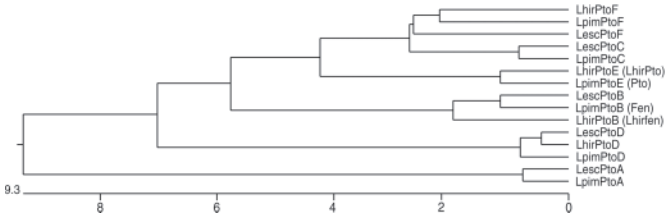

Fig. 3. Relationships among the *Pto* gene family. Phylogenetic tree based on nucleotide sequence identity comparing *LhirPto* genes with *Pto* gene family members from Rio Grande-PtoR (*LpimPto,* GenBank accession no. AF220602) and VFNT Cherry (*LescPto,* GenBank accession no. AF220603). Scale units represent the number of substitution events.

identical to both *LescPtoF* and *LpimPtoF. LescPtoF* was previously isolated from *L. esculentum* line Ailsa Craig and reported to be the ortholog (susceptible allele) of *Pto* (26). Recent data, however, show the *L. esculentum* line VFNT Cherry does not contain a *Pto* ortholog (GenBank accession no. AF220603). It is possible, therefore, that in Ailsa Craig *LescPtoF* is actually a *Pto* paralog that is directly adjacent to the location where a *Pto* ortholog would be if it were present in the VFNT haplotype *Pto* gene cluster.

We did not find *L. hirsutum* cDNA clones corresponding to two predicted open reading frames (*PtoA* and *PtoC*) that are present in both RG-PtoR and VFNT. It is possible that the *Pto* locus has undergone rearrangements since the *L. hirsutum* species diverged, resulting in loss of these *Pto* paralogs. Previous studies have suggested that such rearrangements have taken place within the *L. esculentum Pto* locus, resulting in the deletion of a *Pto* family member (18). However, it is more likely that these genes are simply not expressed in leaf tissue, from which our cDNA library was derived.

***LhirPto* Encodes an Active Kinase That Elicits an *avrPto*-Dependent Hypersensitive Response (HR).** Gene-for-gene resistance often manifests as rapid, localized cell death, termed the HR, that occurs at the site of attempted pathogen ingress. Both tomato and tobacco cells undergo an HR when the *Pto* resistance gene and the *avrPto* avirulence gene are expressed within the same cell. Previous studies also demonstrated that for an *avrPto*-dependent HR to occur, the Pto kinase must possess autophosphorylation activity (4, 5).

To determine whether the LhirPto proteins have autophosphorylation activity we expressed each one as a GST fusion and performed *in vitro* kinase assays. Autoradiography revealed that LhirPto, Lhirfen, and LhirPtoD each autophosphorylated (Fig. 4A). We were unable, however, to detect autophosphorylation by LhirPtoF. Comparing LhirPtoF with other functional protein kinases revealed that LhirPtoF contains a serine residue (Ser-45) in a position normally occupied by an invariant glycine in the nucleotide-binding motif (33). This substitution might be responsible for the observed lack of autophosphorylation activity.

Pto recognition specificity for AvrPto is highly specific as Fen, which shares 87% amino acid similarity with Pto, does not elicit an *avrPto*-dependent HR. A threonine residue (Thr-204) located within the catalytic domain of Pto determines recognition specificity of the kinase for AvrPto (8). Mutation of Thr-204 in Pto abolishes its ability to elicit an HR when it is coexpressed with *avrPto*. Interestingly, LhirPto is the only active LhirPto kinase to contain a threonine in the position corresponding to Thr-204, suggesting this protein may elicit an *avrPto*-dependent HR. To test this possibility, we placed each *L. hirsutum Pto* gene under control of the cauliflower mosaic virus 35S promoter and used *Agrobacterium* to transiently express them with *avrPto* in leaves of *Nicotiana benthamiana*. Indeed, expression of *LhirPto* with *avrPto* in *N. benthamiana* induced an HR 3–5 days after

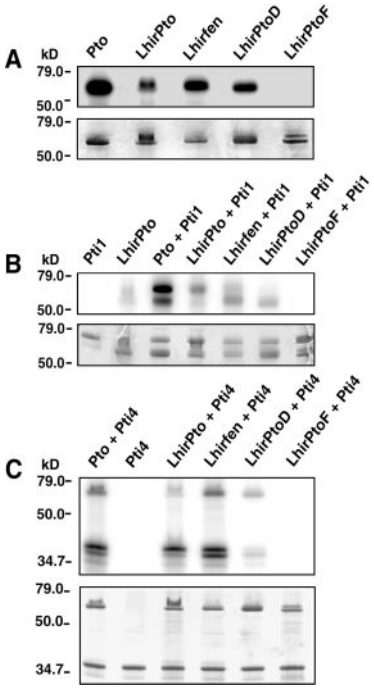

Fig. 4. Phosphorylation assays of the LhirPto proteins. (*A*) Pto and the LhirPto proteins were expressed in bacteria as GST fusions and purified on glutathione-agarose. Each protein (2 μg) was incubated in a kinase reaction with [γ-^{32}P]ATP, separated by SDS/PAGE, and analyzed by autoradiography. (*Upper*) Autoradiogram of autophosphorylated LhirPto proteins. (*Lower*) Coomassie blue-stained gel. The locations of protein standards used to estimate molecular masses are indicated in kilodaltons (kD). (*B*) Assay to test phosphorylation of Pti1 by LhirPto proteins. GST-LhirPto proteins (2 μg) were incubated with 2 μg of purified kinase-deficient GST-Pti1-(K69N) in the presence of [γ-^{32}P]ATP, separated by SDS/PAGE, and analyzed by autoradiography. (*Upper*) Autoradiogram of phosphorylated proteins. (*Lower*) Coomassie blue-stained gel. (*C*) Assay to test phosphorylation of Pti4 by LhirPto proteins. GST-LhirPto proteins (2 μg) were incubated with His-tagged Pti4 (2 μg) in the presence of [γ-^{32}P]ATP, separated by SDS/PAGE, and analyzed by autoradiography. (*Upper*) Autoradiogram of phosphorylated proteins. (*Lower*) Coomassie blue-stained gel.

infiltration identical to coexpression of *Pto* and *avrPto* (Table 2; Fig. 6, which is published as supplemental data on the PNAS web site at www.pnas.org). Expression of either *LhirPto* or *avrPto* alone did not elicit the HR. *Lhirfen*, *LhirPtoD,* and *LhirPtoF* were unable to elicit an HR when expressed either with or without *avrPto*. These results indicate that *LhirPto* is the functional *Pto*

Table 2. *Agrobacterium*-mediated transient expression assays of the *LhirPto* genes

Gene	HR
LhirPto	–
avrPto	–
Pto/*avrPto*	+
LhirPto/*avrPto*	+
Lhirfen/*avrPto*	–
LhirPtoD/*avrPto*	–
LhirPtoF/*avrPto*	–

The indicated genes were placed under control of the cauliflower mosaic virus 35S promoter and cloned into the pBTEX plasmid. These constructs were transformed into *Agrobacterium tumefaciens* strain EHA105. *A. tumefaciens* strains were induced with acetosyringone and syringe-infiltrated into mature *Nicotiana benthamiana* leaves. Leaves were scored for HR 4 days after infiltration. + indicates that total tissue collapse occurred in the infiltrated tissue; – indicates that no tissue collapse was observed.

Table 3. Physical interactions of the LhirPto proteins with AvrPto and Pto-interacting proteins

	AvrPto	Pti1	Pti4	Pti5	Pti6
Pto	++++	+++	++	++	+++
LhirPto	++++	−	++	+++	+++
Lhirfen	−	−	−	−	−
LhirPtoD	−	−	−	++++	++++
LhirPtoF	−	−	−	−	−
Bicoid	−	−	−	−	−

EGY48 yeast cells expressing the LhirPto, Pto, or Bicoid proteins from the bait plasmid pEG202 were transformed with AvrPto, Pti1, Pti4, Pti5, or Pti6 in the prey plasmid pJG4-5. Ten independent transformants for each bait–prey combination were grown on galactose medium containing X-gal and lacking uracil, histidine, and tryptophan to test for *lacZ* reporter gene activation. Representative colonies were scored on the following scale: −, completely white; ++++, dark blue.

gene from *L. hirsutum* var. *glabratum* and that *Lhirfen*, *LhirPtoD*, and *LhirPtoF* do not mediate *avrPto*-specific resistance.

LhirPto Physically Interacts with AvrPto and Pti Proteins. Only Pto alleles which interact with AvrPto in a yeast two-hybrid system can mediate *avrPto*-specific resistance to *P. syringae* (4, 5, 34). We tested each *LhirPto* gene in the yeast two-hybrid system to determine whether the proteins encoded by them could interact with AvrPto. We cloned each *LhirPto* gene in a bait plasmid and performed Western blotting to confirm that each LhirPto-LexA fusion protein was expressed in yeast (data not shown). After confirming that none of the bait constructs activated the *lacZ* reporter gene on their own, we transformed each yeast strain with an AvrPto prey construct and tested for *lacZ* activation (5). Only LhirPto activated the *lacZ* reporter gene when expressed with the AvrPto prey, indicating that LhirPto specifically interacts with AvrPto (Table 3).

Pti1, *Pti4*, *Pti5*, and *Pti6* encode putative downstream members of the Pto signaling pathway (27, 28). We next tested whether the products of the *LhirPto* genes could interact with these signaling molecules. LhirPto activated *lacZ* expression when it was coexpressed with Pti4, Pti5, or Pti6, indicating that LhirPto physically interacts with these transcription factors (Table 3). Surprisingly, LhirPto did not appear to interact with the Pti1 kinase. Neither Lhirfen nor LhirPtoF interacted with any of the Pti proteins tested. LhirPtoD was unable to interact with Pti1 or Pti4 but did interact with Pti5 and Pti6, suggesting that the residues of Pto (and LhirPto) that are required for interaction with these proteins are conserved in this kinase.

LhirPto Phosphorylates Pti1 and Pti4. We have reported previously that the Pto kinase phosphorylates both Pti1 and Pti4 *in vitro* (27, 29). Phosphorylation of Pti1 occurs on residue Thr-233, which is present in the activation domain of Pti1 (34). Thr-233 is also required for the interaction of Pto and Pti1 in a yeast two-hybrid assay, and phosphorylation of Pti1 by Pto may be required for downstream signaling mediated by Pti1. Phosphorylation of Pti4 by Pto enhances the affinity of Pti4 for *PR* gene promoter elements in mobility-shift assays (29). We performed *in vitro* kinase assays to determine whether the LhirPto proteins could phosphorylate kinase-deficient Pti1(K69N) or Pti4. Although we were unable to detect interaction of LhirPto with Pti1 in a yeast two-hybrid assay, LhirPto did phosphorylate Pti1 *in vitro* (Fig. 4*B*). The ability to phosphorylate Pti1 was specific to LhirPto, as none of the other LhirPto proteins phosphorylated Pti1. LhirPto also phosphorylated Pti4 *in vitro* (Fig. 4*C*). However, unlike the phosphorylation of Pti1, the ability to phosphorylate Pti4 was not specific to LhirPto as the Lhirfen protein also strongly phosphorylated Pti4.

Discussion

We have presented evidence that, in common with the product of the *Pto* gene isolated from *L. pimpinellifolium*, the *L. hirsutum* Pto kinase interacts specifically with the *Pseudomonas* AvrPto protein and mediates an *avrPto*-specific HR in an *N. benthamiana* transient expression assay. We have further demonstrated that the LhirPto kinase interacts with downstream members of the Pto pathway, suggesting that these signaling components are also conserved among different tomato species. These results indicate that the specificity of the *Pto* gene for the *Pseudomonas* AvrPto protein evolved before the divergence of *L. hirsutum* and *L. pimpinellifolium*.

Evidence for an Ancient Origin of Pto Kinase Recognition Specificity. The *Pto* locus itself appears to be of ancient origin. *Pto* homologs map to syntenic regions in tomato, potato, and pepper, and the genome organization of the *Pto* locus is mostly conserved between *L. esculentum* and *L. pimpinellifolium,* indicating that the locus predates the divergence of these species (35, 36). Despite many decades of disease resistance breeding, however, *R* genes have yet to be identified that map to the *Pto* locus in potato or pepper. This observation indicates that a role in pathogen recognition and disease resistance for the *Pto* gene family might have evolved subsequent to the divergence of the *Lycopersicon* lineage or, alternatively, has since been lost from potato and pepper.

We earlier confirmed that the *L. hirsutum* accession PI134418 expresses *avrPto*-specific resistance and mapped that resistance to the *Pto* locus (22). We have now demonstrated that a member of the *Pto* gene family in PI134418, *LhirPto*, encodes a protein that bears remarkable sequence identity and functional similarity to the *L. pimpinellifolium* Pto kinase. The presence of functional *Pto* orthologs in two distantly related tomato species suggests that *Pto* evolved the capability to recognize the AvrPto protein before the divergence of *L. pimpinellifolium* and *L. hirsutum*. We cannot, of course, exclude the possibility that *Pto* may possess an additional function that predates AvrPto recognition and is the driving force behind the conservation of *Pto*. However, the absence of *Pto* in *L. esculentum* and other *Lycopersicon* species (see below) demonstrates that any putative secondary function is dispensable. It seems more likely, therefore, that Pto and AvrPto coevolved and that AvrPto recognition provided the impetus to conserve the resistance function of *Pto.*

In contrast to AvrPto recognition specificity, sensitivity to fenthion, which has been identified only in *L. pimpinellifolium*, appears to be more recent. *Fen* orthologs have been identified in both *L. esculentum* and *L. hirsutum* and share 97% and 95% nucleotide identity with *Fen*, respectively. However only the Fen kinase is capable of recognizing fenthion. Thus relatively few (and perhaps recent) changes in the Fen amino acid sequence appear to underlie the specificity involved in this recognition mechanism. Similarly, single amino acid substitutions within the Pto and Fen kinases can confer or eliminate recognition of AvrPto (8, 34, 37). Despite the apparent delicate nature of recognition specificity in the kinase class of *R* gene, 17 amino acid differences that have arisen to distinguish Pto and LhirPto do not disrupt Pto disease resistance function. This further suggests that there has been selective pressure to maintain AvrPto recognition specificity.

It is interesting to note that *Pst* resistance originating in the cultivated tomato, *L. esculentum*, has yet to be identified. Although some *L. esculentum* varieties express *avrPto*-specific *Pto*-mediated resistance, RFLP analyses show that this resistance likely originated in *L. pimpinellifolium* (ref. 38; G.B.M., unpublished data). Furthermore, it has been reported recently that *Pto* orthologs are entirely absent from two different *L. esculentum* haplotypes (18). Why would a gene that has retained its recog-

nition specificity over much of *Lycopersicon* evolution be absent from an entire species? A simple explanation is that during the course of domestication of wild *L. esculentum* a genetic bottleneck occurred that resulted in the selection of *L. esculentum* lines that, by chance alone, lacked the *Pto* gene. An alternative possibility is that in the absence of *Pst* expressing the *avrPto* gene there is a fitness cost associated with the *Pto* locus in *L. esculentum* that has resulted in selection against lines containing *Pto* either in nature or in cultivated fields (39). Tanksley and colleagues (25) compared 17 processing traits between *L. esculentum* near-isogenic lines with and without the introgressed *Pto* locus. They concluded that with respect to these 17 traits, *Pto*/*Pto* plants were not significantly different from *pto*/*pto* plants. Therefore it seems unlikely that the *Pto* locus exacts a fitness cost that might result in negative selection. It remains a possibility that the *Pto* locus confers deleterious effects on fitness that might be difficult to detect experimentally.

***LhirPto* and *Pto* Mediate Resistance Through a Conserved Signaling Pathway.** We have demonstrated that in an *L. esculentum* background *LhirPto* mediates *avrPto*-specific disease resistance that is indistinguishable from that conferred by the *Pto* gene. We infer from this observation that *Pto* and *LhirPto* use the same signaling components to effect *Pseudomonas* resistance in *L. esculentum*. Our present study demonstrates that Pto and LhirPto do indeed interact with a similar array of downstream components. Previous investigations have identified *L. esculentum* signaling molecules with which Pto interacts both physically and biochemically (27–29). In common with the Pto kinase, LhirPto interacts with the transcription factors Pti4, Pti5, and Pti6 in the yeast two-hybrid system and phosphorylates Pti4 *in vitro*. On the basis of these results we speculate that, similar to Pto, the interaction and phosphorylation of Pti4 by LhirPto enhances the binding of Pti4 to the GCC box cis element to activate *PR* gene expression (29). Although we were unable to detect an interaction between the LhirPto and Pti1 proteins in the yeast two-hybrid system, LhirPto retains the ability to specifically phosphorylate Pti1 in an *in vitro* kinase assay. The structural and functional similarities that exist between LhirPto and Pto and the phosphorylation of Pti1 by LhirPto *in vitro* suggest that LhirPto does use the Pti1 kinase in defense signaling. Taken together, our data suggest that *L. esculentum, L. pimpinellifolium,* and the more distantly related *L. hirsutum* share a common signaling pathway that mediates resistance to bacterial speck disease. Considering that the *Pto* and *LhirPto* genes also function in *N. benthamiana,* this pathway likely predates speciation within the *S*olanaceae.

An Ancient Origin of AvrPto Recognition Specificity Supports the Trench Warfare Hypothesis. Several recent reports have presented evidence that the LRR class of *R* genes evolve rapidly to develop new recognition specificities (16–19, 40). These reports indicate that the LRR domain is under diversifying selection to create novel recognition capabilities in response to pathogen pressure and thus support an "arms-race" model for *R–avr* gene evolution (41). In contrast, a comparison of nucleotide sequences from the *RPM1* locus of different *Arabidopsis* ecotypes suggested that some *R–avr* gene pairs have coexisted for millions of years (20). According to the "trench warfare" hypothesis proposed in that study, gene-for-gene interactions are ancient and polymorphism at *R* gene loci is governed by frequency-dependent selection as a function of both disease frequency and a "cost of resistance" associated with the *R* gene.

We have reported genetic and molecular analyses demonstrating that *Pto* orthologs from *L. hirsutum* and *L. pimpinellifolium* mediate identical recognition specificity for AvrPto. These findings indicate that AvrPto recognition arose before these two species diverged and suggest that Pto and AvrPto have coexisted for millions of years. Surveys of wild tomato germ plasm have previously shown that not all *Lycopersicon* accessions are resistant to *Pst,* and recent sequencing of the *Pto* locus in VFNT Cherry found that a *Pto* ortholog is not even present in that *L. esculentum* haplotype (18, 42, 43). Thus significant functional polymorphism exists at the *Pto* locus in *Lycopersicon*. In conjunction with the ancient origins of the Pto–AvrPto interaction, the polymorphic nature of the *Pto* locus supports the trench-warfare hypothesis in which the "rise and fall" of a specific *R* gene maintains variation for disease resistance in a plant population (20).

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