Cell, Vol. 83, 925–935, December 15, 1995, Copyright © 1995 by Cell Press

The Tomato Gene *Pti1* Encodes a Serine/Threonine Kinase That Is Phosphorylated by Pto and Is Involved in the Hypersensitive Response

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Summary

The *Pto* gene encodes a serine/threonine kinase that confers resistance to bacterial speck disease in tomato. Using the yeast two-hybrid system, we identified a second serine/threonine kinase, Pto-interacting 1 (Pti1), that physically interacts with Pto. Cross-phosphorylation assays revealed that Pto specifically phosphorylates Pti1 and that Pti1 does not phosphorylate Pto. Fen, another serine/threonine kinase from tomato that is closely related to Pto, was unable to phosphorylate Pti1 and was not phosphorylated by Pti1. Expression of a *Pti1* transgene in tobacco plants enhanced the hypersensitive response to a P. syringae pv. tabaci strain carrying the avirulence gene *avrPto*. These findings indicate that Pti1 is involved in a Pto-mediated signaling pathway, probably by acting as a component downstream of Pto in a phosphorylation cascade.

Introduction

Plants are equipped with a variety of mechanisms to defend themselves from pathogens (Lamb et al., 1989; Lamb, 1994). These defense responses include a rapid induction of localized necrosis at the site of infection (the hypersensitive response [HR]), increased expression of defense-related genes, production of anti-microbial compounds, lignin formation, and the oxidative burst (Cutt and Klessig, 1992; Goodman and Novacky, 1994; Levine et al., 1994; Mehdy, 1994). In many plant–pathogen interactions, defense responses are activated upon recognition of a pathogen carrying a specific avirulence (*avr*) gene by a plant host containing a corresponding resistance (*R*) gene (Flor, 1971; Keen, 1990). Elucidation of the molecular mechanisms by which plant defense systems are activated after specific recognition of a pathogen offers great potential for increasing the effectiveness of natural plant resistance by genetic engineering (Keen et al., 1993).

Intense efforts in the past decade have led to several recent successes in cloning *R* genes conferring resistance to a wide spectrum of plant pathogens, including viruses, bacteria, and fungi (Dangl, 1995; Martin, 1995; Staskawicz et al., 1995). Most of the *R* genes cloned to date confer disease resistance in a "gene-for-gene" manner by recognizing specific avirulent strains of the corresponding pathogen (Flor, 1971). It was expected that these *R* genes would encode components of signaling pathways that ultimately lead to defense responses, and this appears to be the case. For example, the *Pto* gene, which confers resistance to bacterial speck disease in tomato, encodes a protein kinase (Loh and Martin, 1995; Martin et al., 1993b).

Other recently cloned *R* genes involved in gene-for-gene resistance also encode proteins with features indicative of signal recognition and transduction. For example, the *Cf-9*, *RPS2*, *L6*, and *N* genes all encode proteins that contain a region of leucine-rich repeats suggestive of ligand binding or direct protein–protein interactions (Bent et al., 1994; Jones et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994; Lawrence et al., 1995). In addition, RPS2, N, and L6 proteins share significant homology and contain putative nucleotide-binding sequences, implying functional importance for these sequences (Traut, 1994). The Cf-9 protein is unique among the characterized *R* gene products in that it possesses a signal peptide sequence, a large leucine-rich repeat, and a transmembrane domain (Jones et al., 1994). A major portion of this protein might be extracellular and could function directly as a receptor for the fungal avr9 polypeptide. The L6 protein from flax also contains a signal peptide, and it may attach to a plant cell membrane (Lawrence et al., 1995). The remaining *R* gene products, including Pto, are thought to be cytoplasmic proteins, and the mechanism(s) by which they respond to a pathogen elicitor molecule remains uncertain.

In tomato, the *Pto* locus confers resistance specifically to Pseudomonas syringae pv. tomato strains expressing the avirulence gene *avrPto* (Martin et al., 1993b). It is assumed that *avrPto* mediates the production of a specific "elicitor," although no such molecule has yet been identified. Interestingly, most tomato cultivars that carry bacterial speck resistance rapidly develop small necrotic lesions upon exposure to the insecticide fenthion (Martin et al., 1993a). The *Fen* gene, which controls this sensitivity, is located close to the *Pto* gene on chromosome 5 (Martin et al., 1994). The similarity between fenthion-induced necrosis and the pathogen-induced HR raises the intriguing possibility that fenthion is structurally similar to the elicitor molecule produced by the avirulent bacterium (Martin et al., 1994). Mutagenesis of a bacterial speck–resistant tomato line has identified another gene near *Pto*, named *Prf*, that is required for both *Pto*-mediated resistance and fenthion sensitivity (Salmeron et al., 1994). *Pto* and *Fen* were isolated by map-based cloning and were found to belong to a clustered gene family of five to seven members (Martin et al., 1993b, 1994). The Pto protein shares 80% identity (87% similarity) with Fen. Introduction of a *Pto* transgene into a susceptible tomato cultivar or into tobacco results in a marked increase in resistance to *avrPto*-expressing strains of P. syringae tomato or P. syringae tabaci, respectively (Martin et al., 1993b; Rommens et al., 1995b; Thilmony et al., 1995). As with other *avr* gene–*R*

gene associations, there is evidence that functional *Pto* homologs exist in other plant species, including soybean and tobacco (Ronald et al., 1992; Thilmony et al., 1995). Furthermore, *Pto*-related sequences have been detected in a wide spectrum of plant species, ranging from dicots to monocots (Martin et al., 1993b). Thus, a signaling pathway involving a *Pto*-like gene may be widely conserved in the plant kingdom.

Pto and Fen are functional protein kinases that specifically phosphorylate serine and threonine residues (Martin et al., 1993b, 1994; Loh and Martin, 1995; Rommens et al., 1995a). We have therefore hypothesized that a protein phosphorylation cascade is involved in Pto-mediated disease resistance. The testing of this hypothesis and further delineation of the Pto signaling pathway would benefit from the identification of proteins that interact with Pto. As an alternative approach to mutagenesis, which to date has uncovered only two loci involved in tomato bacterial speck resistance (*Pto* and *Prf*), we utilized the yeast two-hybrid system to clone directly genes encoding proteins that interact physically with Pto (Gyuris et al., 1993). Here we report the isolation of cDNAs encoding Pto-interacting (Pti) proteins. One of these genes, *Pti1*, encodes a functional protein kinase with serine/threonine specificity. Pti1 is a specific substrate of the Pto kinase when assayed in vitro. Furthermore, expression of a *Pti1* transgene in tobacco results in an accelerated HR when the plants are challenged with the tobacco pathogen P. syringae tabaci expressing *avrPto*. These data provide strong evidence for a second component in the Pto-mediated signaling pathway and confirm a central role for a phosphorylation cascade in gene-for-gene plant disease resistance.

Results

Two-Hybrid Screen for Pto-Interacting Proteins

The entire open reading frame, *ORF1*, contained in *Pto* cDNA clone CD186 (Martin et al., 1993b) was fused in-frame to the C-terminus of the DNA-binding domain of LexA in the "bait" plasmid pEG202 and transformed into yeast strain EGY48 containing two reporter genes, *LexAop–LEU2* and *LexAop–lacZ* (Gyuris et al., 1993). The plasmid pEG202–Pto did not activate transcription of the two reporter genes, and a repression assay indicated that the LexA–Pto fusion protein entered the yeast nucleus (Gyuris et al., 1993; data not shown). For the "prey" library, we prepared cDNA from mRNA isolated from a bacterial speck–resistant tomato line, Rio Grande–PtoR, that had been inoculated with avirulent P. syringae tomato strain T1/*avrPto*. The cDNA inserts were cloned into a site downstream of the acidic transcription activation domain in plasmid pJG4-5 (Gyuris, 1993; see Experimental Procedures).

An initial screen of the library recovered approximately 1000 leucine-prototrophic colonies, and we further characterized 500 fast-growing colonies for β-galactosidase activity by streaking individual colonies onto galactose medium containing X-Gal. Of the colonies, 149 appeared dark blue, and they belonged to ten distinct classes as indicated by cross-hybridization experiments. One class of cDNAs, designated *Pti1*, was fully characterized and is described here.

The *Pti1* class contained three clones, each containing a 1.4 kb insert. We retransformed one of the clones into strain EGY48 to test for autoactivation of the reporter genes and into other yeast strains to examine the specificity of the interaction. Pti1 expression alone in EGY48 did not activate transcription of the reporter genes. Coexpression of Pti1 with an arbitrary bait protein, Bicoid, or coexpression of Pto with several prey proteins (Pelle, Tube, Dorsal, and Raf; Großhans et al., 1994) similarly did not activate reporter gene transcription (Figure 1; D. Halterman and G. B. M., unpublished data). Coexpression of Pti1 and the Pto bait protein in EGY48 produced leucine prototrophy and cleavage of X-Gal (Figure 1). Both phenotypes were dependent upon growth on galactose medium, indicating that expression of Pti1 was required for the activation of the reporter genes. Thus, *Pti1* encodes a protein that physically interacts with the Pto kinase in the yeast two-hybrid system. We next tested possible interaction between Pti1 and Fen in the yeast two-hybrid system. In contrast with the yeast cells expressing both Pto and Pti1, the yeast cells expressing Fen and Pti1 had a low level of the β-galactosidase activity (data not shown). Therefore, the interaction between Pto and Pti1 is highly specific. The Pto protein contains a putative myristoylation site at its N-terminus (Martin et al., 1993b), and myristoylation facilitates protein–protein interaction in some systems (Towler and Gordon, 1988). However, this site is altered in the LexA–Pto fusion protein and therefore must not be required for the interaction of Pto and Pti1.

Pti1 Is a Member of a Gene Family That Is Conserved in Plants

The entire nucleotide sequence was determined for the 1.4 kb *Pti1* cDNA clone described above. Partial nucleotide sequence analysis showed that the other two *Pti1* class

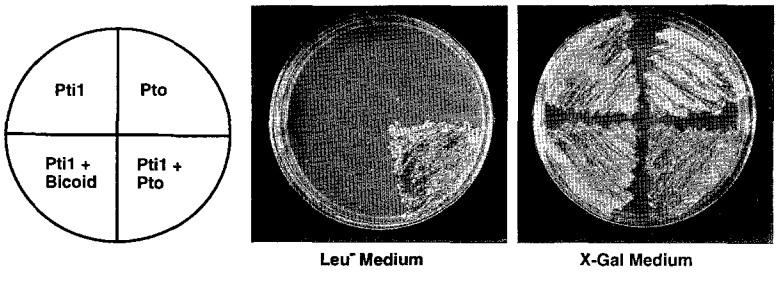

Figure 1. Interaction of Pto with Pti1

EGY48 yeast cells containing Pti1 (in pJG4-5), Pto (in pEG202), Pto and Pti1, or Bicoid and Pti1 (in pEG202 and pJG4-5, respectively) were grown on galactose medium lacking uracil and leucine (middle) or galactose X-Gal medium lacking uracil (right). The media in both plates contained histidine and tryptophan to permit growth of all four strains. The plates were incubated at 30°C for 3 days.

cDNA clones were identical. As expected, an open reading frame encoded by this cDNA was fused in-frame to the transcriptional activation domain of pJG4-5. To determine the size of the transcript corresponding to this cDNA, we radiolabeled the 1.4 kb cDNA insert and probed a Northern blot containing poly(A)$^+$ RNA isolated from leaves of both bacterial speck–resistant and susceptible tomato plants (Figure 2A). A 1.6 kb transcript was detected in both tomato lines, indicating that the 1.4 kb *Pti1* cDNA represented a partial clone of the *Pti1* transcript (Figure 2A). A full-length cDNA clone was isolated by screening a tomato cDNA library using the 1.4 kb *Pti1* cDNA as a probe. We isolated 51 positive plaques in this screen, comprised of

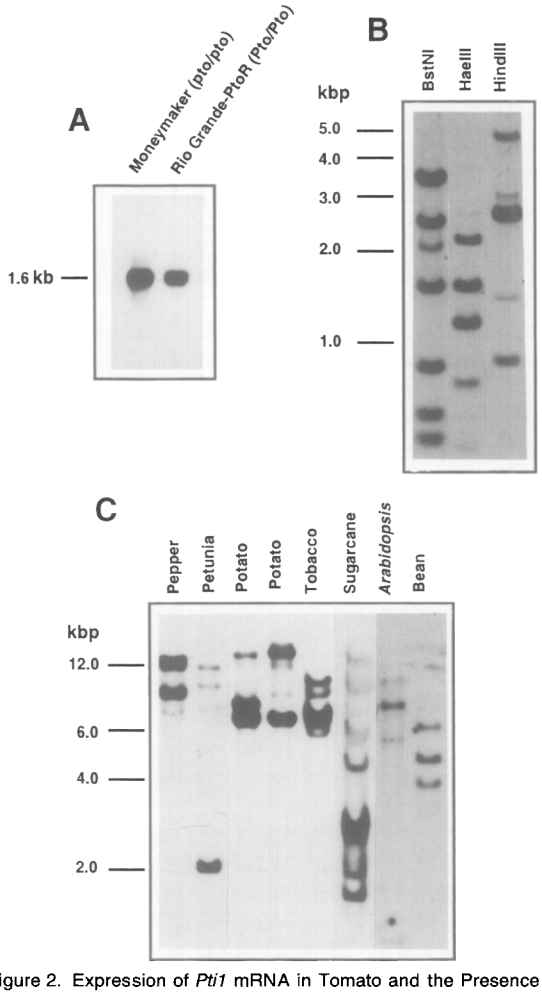

Figure 2. Expression of *Pti1* mRNA in Tomato and the Presence of *Pti1*-Related Sequences in Tomato and Other Plant Genomes

(A) Northern blot analysis of *Pti1* transcript in tomato. An RNA gel blot containing 4 μg per lane of poly(A)$^+$ RNA from leaves of tomato cultivars Moneymaker or Rio Grande–PtoR was hybridized to the *Pti1* cDNA probe as described in Experimental Procedures.

(B) DNA gel blot analysis of tomato genomic DNA. Genomic DNA (5 μg per lane) from Rio Grande–PtoR plants was digested with the indicated restriction enzymes, and the DNA blot was hybridized to the *Pti1* cDNA probe.

(C) DNA gel blot analysis of genomic DNA isolated from other plant species. A genomic DNA gel blot containing EcoRI-digested DNA from different plant species was hybridized to the *Pti1* probe as described previously (Martin et al., 1993b).

26 strongly hybridizing and 25 weakly hybridizing clones. Ten strongly hybridizing plaques were individually purified, and plasmids containing *Pti1*-homologous inserts were isolated. XbaI and XhoI digests of these clones revealed that they represented at least two distinct classes of *Pti1* homologs (data not shown). One 1.6 kb cDNA clone had the same restriction enzyme sites as the original *Pti1* cDNA. Further analysis showed that this 1.6 kb cDNA shared the identical nucleotide sequence with the original 1.4 kb clone and was a full-length clone of *Pti1*.

Southern blot hybridization using the 1.4 kb cDNA as a probe detected multiple fragments in tomato DNA (Figure 2B). With lower stringency washes, *Pti1* hybridized to several additional bands on tomato genomic DNA gel blots (data not shown). These results, together with the isolation of at least two classes of *Pti1* clones from the cDNA library, suggest that *Pti1* is a member of a gene family. Hybridization of a radiolabeled *Pti1* cDNA probe to a DNA gel blot of genomic DNA from tobacco, pepper, potato, Petunia, Arabidopsis, bean, and sugarcane detected homologous sequences in all of these plant species (Figure 2C). A stronger hybridization signal was detected among the Solanaceous species, of which tomato is a member, which probably indicates a greater conservation of *Pti1*-related sequences within this plant family.

Pti1 Is Similar to Various Protein Kinases

The 1.6 kb full-length cDNA clone of Pti1 contains a 1110 bp open reading frame (Figure 3), a 101 bp 5′ untranslated region, and a 351 bp 3′ untranslated region including a poly(A) tail. The first ATG of the open reading frame starts at 102 bp and is preceded by an in-frame stop codon at the −6 to −4 position and a purine (G) at the −3 position. This is a favorable context for initiating translation in eukaryotes (Kozak, 1989). Translation of this open reading frame would produce a protein of 370 amino acids with a molecular mass of 40.7 kDa. The first seven amino acids of Pti1 apparently are not required for interaction with Pto, because the original *Pti1* cDNA clones isolated from the two-hybrid screen lacked the nucleotides corresponding to these residues.

A search of current databases with the deduced Pti1 amino acid sequence revealed significant homology to various protein kinases. The putative kinase catalytic domain starts approximately 70 amino acid residues after the first methionine in Pti1 and contains the 11 subdomains that are typical of protein kinases (Figure 3; Hanks and Quinn, 1990). The majority of protein kinases share 15 invariant amino acid residues in their catalytic domain; however, two of these invariant residues are not conserved in Pti1. The conserved glutamine in subdomain III is substituted with a glutamate at position 111 in Pti1, and the conserved glycine in subdomain VII is substituted with an aspartate at position 218 in Pti1.

The protein kinases with greatest similarity to Pti1 are MHK and APK1 from Arabidopsis and the tomato *Pto* and *Fen* gene products (Figure 3). The Pti1 amino acid sequence shares 38.2% overall identity and 43.4% identity within the kinase domain to MHK and 37.6% overall iden-

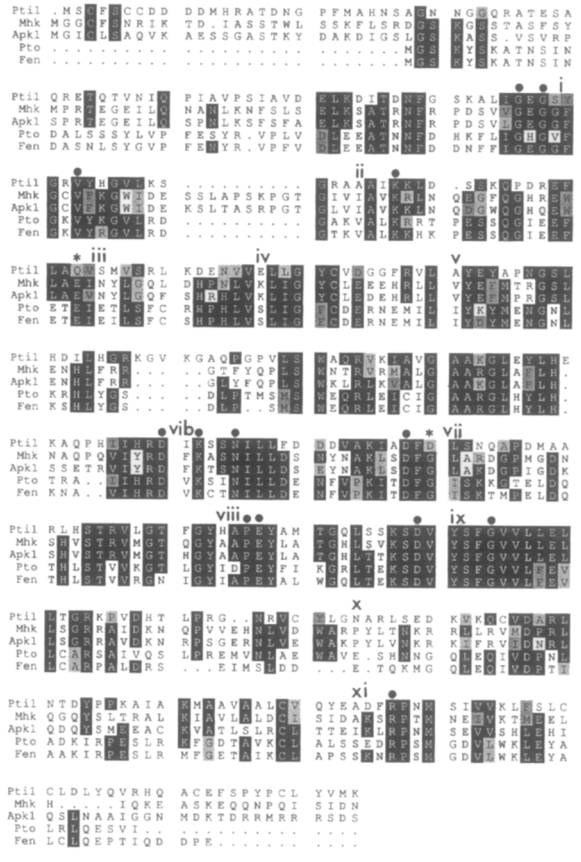

Figure 3. Alignment of Pti1 Amino Acid Sequence with Four Other Protein Kinases from Plants

The kinases include MHK and APK1 from Arabidopsis (Moran and Walker, 1993; Hirayama and Oka, 1992), Pto, and Fen from tomato (Martin et al., 1993b, 1994). The Pretty Box program (GCG package, version 7.0) was used to create the best alignment. Amino acids identical in at least three of the sequences are highlighted in black, and conservative substitutions are stippled. The 11 subdomains conserved in protein kinases are indicated with Roman numerals above the sequences. The invariant residues conserved in all protein kinases are labeled with bullets (Hanks and Quinn, 1990). The invariant residues that are conserved in other protein kinases but not in Pti1 are marked with asterisks.

tity and 43.1% identity within the kinase domain to APK1. MHK and APK1 were isolated based solely on their conserved domains, and a role for either kinase is unknown. Pti1 shows less sequence similarity with Pto (36.4% overall identity and 38.5% identity within the kinase domain) and Fen (36.4% overall identity and 38.8% identity in the kinase domain).

Products of several *R* genes contain leucine-rich repeats, a nucleotide-binding site, or a transmembrane domain, and it is possible that proteins similar to these *R* gene products may interact with Pto (Dangl, 1995; Martin, 1995). However, none of these features is present in the Pti1 protein. In addition, unlike Pto and Fen, Pti1 lacks a putative myristoylation sequence. Based on these observations, Pti1 is probably a cytoplasmic kinase.

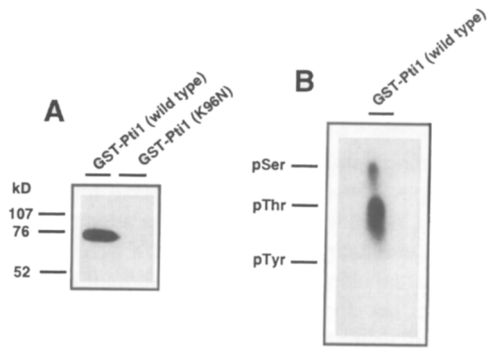

Figure 4. Pti1 Is a Functional Protein Kinase with Serine/Threonine Specificity

(A) Autophosphorylation of GST–Pti1. A wild-type GST–Pti1 fusion protein and the kinase-deficient mutant GST–Pti1(K96N) were overexpressed and purified from E. coli, immobilized on glutathione–agarose beads, incubated with [γ-^{32}P]ATP in kinase buffer, electrophoresed on SDS–polyacrylamide gels, and exposed to X-ray film.

(B) Phosphoamino analysis of ^{32}P-labeled autophosphorylated GST–Pti1. The locations of phosphoamino acid standards of serine, threonine, and tyrosine are as indicated.

Pti1 Is a Functional Serine/Threonine Protein Kinase

To determine whether Pti1 encodes a functional protein kinase, the original 1.4 kb *Pti1* cDNA was fused in-frame to the C-terminus of the bacterial glutathione S-transferase (GST) and expressed in E. coli. The fusion protein was affinity purified and shown to have the expected molecular mass of 70 kDa (Figure 4A). Incubation of the purified fusion protein with [γ-^{32}P]ATP in an in vitro kinase assay showed that Pti1 was capable of strong autophosphorylation (Figure 4A). The highly conserved lysine residue in subdomain II is required for activity in most protein kinases. We replaced this lysine with asparagine using site-directed mutagenesis and assayed the mutant protein for kinase activity. The K96N mutation completely abolished the autophosphorylation activity of Pti1. Thus, although Pti1 contains substitutions at two highly conserved residues in subdomains III and VII, it encodes a highly active protein kinase.

To determine the amino acid specificity of the Pti1 kinase, autophosphorylated GST–Pti1 fusion protein was hydrolyzed with HCl, and the constituent amino acids were separated by thin-layer chromatography. Serine and threonine residues were phosphorylated in GST–Pti1 (Figure 4B).

Pti1 Does Not Phosphorylate Pto or Fen

The physical interaction of Pto with Pti1 and the fact that both encode functional protein kinases raised the possibility that these proteins represent two sequential steps in a phosphorylation cascade. We first examined the possible phosphorylation of Pto by Pti1 in an effort to define the relationship of Pti1 and Pto (Figure 5). Because Fen and Pto are structurally similar and may function in a similar way, we also included the Fen protein in our experiments. Previously, both Pto and Fen were expressed as fusions

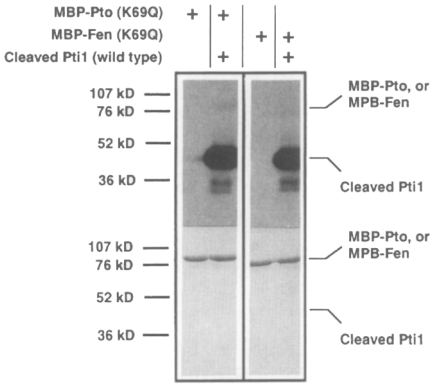

Figure 5. Pti1 Does Not Phosphorylate Pto or Fen

The E. coli–expressed, kinase-deficient protein MBP–Pto(K69Q) or MBP–Fen(K69Q) was immobilized on amylose resin and incubated with or without the cleaved wild-type Pti1 protein in kinase assays. The liquid phase was removed, and proteins remaining on the resin were separated by SDS–PAGE. The autoradiograph (top) shows the phosphorylated proteins and the Coomasie-stained gel (bottom) shows the protein profile.

with the maltose-binding protein (MBP) in E. coli and shown to have kinase activity (Loh and Martin, 1995). To distinguish between cross-phosphorylation and autophosphorylation of Pto or Fen, we used the kinase-deficient fusion proteins MBP–Pto(K69Q) and MBP–Fen(K69Q), in which the invariant lysine residue in subdomain II was substituted with glutamine (Loh and Martin, 1995). The wild-type Pti1, from which the GST portion had been removed by thrombin cleavage, failed to phosphorylate MBP–Pto(K69Q) or MBP–Fen(K69Q) in an in vitro kinase assay, although Pti1 was strongly autophosphorylated (Figure 5).

Pto, but Not Fen, Phosphorylates Pti1 on Serine and Threonine Residues

We next tested the ability of purified Pto and Fen fusion proteins to phosphorylate Pti1 in vitro. We incubated the mutant GST–Pti1(K96N) with the wild-type MBP–Pto or MBP–Fen to distinguish Pti1 autophosphorylation from cross-phosphorylation of Pti1 by Pto or Fen (Figure 6A). GST–Pti1(K96N) did not autophosphorylate, but was strongly phosphorylated when incubated with MBP–Pto. The MBP–Fen fusion protein, however, did not phosphorylate GST–Pti1(K96N). Removal of GST from the GST–Pti1(K96N) fusion protein via thrombin cleavage did not alter the phosphorylation of Pti1(K96N) (Figure 6B).

Pto specifically autophosphorylates on serine/threonine residues (Loh and Martin, 1995), and we expected that Pto would phosphorylate Pti1 on serine/threonine residues. To verify this, we subjected the phosphorylated Pti1(K96N) protein from Figure 6B to phosphoamino analysis. As expected, Pti1 was phosphorylated exclusively on serine and threonine residues by MBP–Pto (Figure 6C). Although there are more serine residues (26) than threonine residues (11) in Pti1, the majority of phosphorylation occurred on threonine residues (Figure 6C).

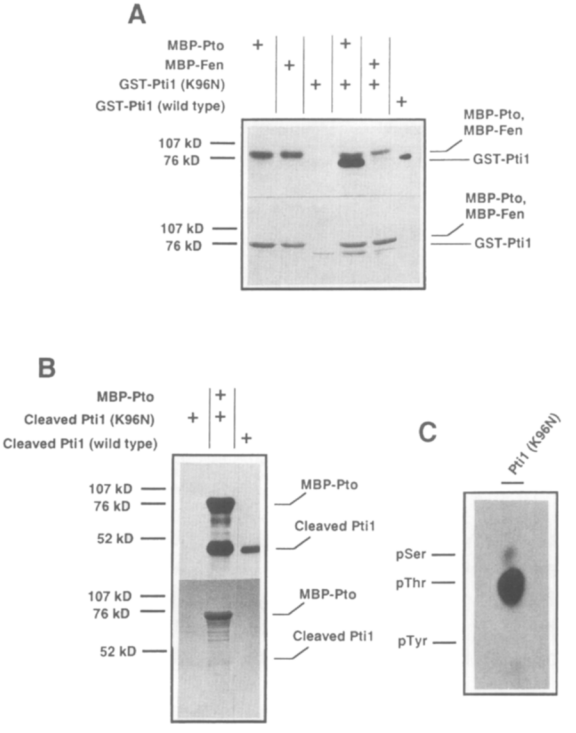

Figure 6. Pti1 is Phosphorylated by Pto but Not Fen

(A) Cross-phosphorylation of Pti1 by Pto, but not Fen. The E. coli–expressed kinase-deficient GST–Pti1(K96N) protein was incubated alone or with the wild-type MBP–Pto or MBP–Fen protein in kinase assays, and the total protein was analyzed by SDS–PAGE and autoradiography. The autoradiograph (top) shows the phosphorylated proteins and the Coomasie-stained gel (bottom) shows the protein profile.

(B) Cross-phosphorylation of cleaved Pti1 by Pto. The cleaved Pti1(K96N) protein was incubated with or without the wild-type MBP–Pto protein in kinase assay in the presence of [γ-^{32}P]ATP and analyzed by SDS–PAGE (bottom) and autoradiography (top).

(C) Phosphoamino analysis of the cleaved Pti1(K96N) that has been phosphorylated by Pto.

A Kinase-Deficient Pto Protein Is Unable to Interact Physically with Pti1

To test whether Pto kinase activity is required for the physical interaction with Pti1, we introduced the cDNA encoding the mutant Pto(K69Q) protein into pEG202 and transformed this plasmid into yeast strain EGY48 containing *Pti1* in prey plasmid pJG4-5. The resulting yeast strain failed to grow in leucine-deficient medium and was unable to activate the *lacZ* reporter gene when grown in galactose medium (Table 1). These observations suggest that phosphorylation of Pto is a prerequisite for the interaction and subsequent cross-phosphorylation of Pti1.

We next tested whether kinase-deficient Pti1 protein was able to interact with wild-type Pto protein. A cDNA insert encoding the mutant Pti1(K96N) was inserted into the plasmid pJG4-5 and introduced into the yeast strain EGY48 containing the wild-type *Pto* gene in plasmid pEG202. The resulting yeast strain was able to grow on galactose medium lacking leucine (Table 1). A slightly lower but similar galactose-inducible β-galactosidase activity was detected in these cells when compared with the

Table 1. Effects of Mutations of Kinase Subdomain II on the Interaction of Pto and Pti1

pEG202 Construct	pJG4-5 Construct	Growth on Leu⁻ Medium[a]	β-Galactosidase Activity[b]
Bicoid	Pti1	No	7
Pto	Pti1	Yes	75
Pto(K69Q)	Pti1	No	4
Pto	Pti1(K96N)	Yes	42

EGY48 yeast cells containing various baits and preys were tested for *LEU2* gene activation on agar plates lacking leucine and *lacZ* gene activation in liquid culture.

[a] At least ten independent transformants were streaked on plates lacking leucine (Leu⁻) and grown for 3 days.

[b] β-Galactosidase activity is expressed as relative units (Reynolds and Lundblad, 1989). Numbers represent the average of three independent transformants, each with four measurements.

yeast strain containing wild-type Pto and wild-type Pti1. These results demonstrate that the lysine residue in the ATP-binding site of Pti1 is not essential for its interaction with Pto. This is consistent with our hypothesis that phosphorylated Pto initiates the interaction of Pto with Pti1.

Expression of a *Pti1* Transgene in Tobacco Plants Enhances Resistance to P. syringae tabaci Carrying *avrPto*

To test the biological relevance of *Pti1* gene expression, we introduced the *Pti1* gene into tobacco cultivar Wisconsin-38 (W-38) under control of a strong constitutive promoter. W-38 contains a functional homolog of *Pto*, which inhibits growth of a P. syringae tabaci strain expressing *avrPto* by 3- to 8-fold in comparison with growth of a virulent P. syringae tabaci strain (Thilmony et al., 1995). Expression of the tomato *Pto* gene in this tobacco cultivar greatly enhances this resistance (Thilmony et al., 1995). Thus, components required for Pto-mediated signaling are functionally conserved between tomato and tobacco. Tobacco is particularly amenable to analysis of the HR, and the conservation of Pto function in this species, therefore, provides an ideal system to test possible in vivo function of Pto-interacting proteins.

The 1.6 kb full-length *Pti1* cDNA was inserted into plasmid pBI121 in the sense orientation under control of the cauliflower mosaic virus 35S promoter. The resulting construct was introduced into W-38 plants using Agrobacterium-mediated transformation. Five independent transgenic plants were regenerated, as confirmed by DNA gel blot analysis (49-2, 49-4, 49-7, 49-14, and 49-16). Analysis of three transgenic lines is shown in Figure 7A. Northern blot analysis indicated that at least two transgenic lines, 49-2 and 49-4, expressed a *Pti1* transcript of the expected size (Figure 7B).

To examine the effect of the *Pti1* transgene on the plant response to pathogen infection, we inoculated the five primary transgenic plants (R0) with a high titer of P. syringae tabaci expressing *avrPto* and found that four plants (49-2, 49-4, 49-7, and 49-16) consistently reacted with a rapid HR 10–12 hr following inoculation (Figure 7C). The HR began with a collapse of the inoculated area, followed by

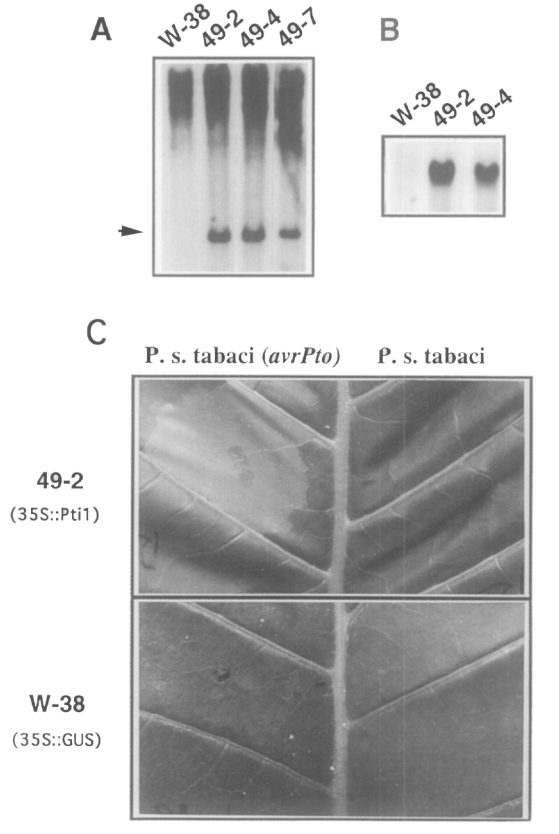

Figure 7. A *Pti1* Transgene Enhances the Tobacco HR to a P. syringae tabaci Strain Expressing *avrPto*

(A) DNA gel blot of transgenic tobacco plants. The DNA blot containing EcoRI-digested genomic DNA from a wild-type W-38 plant or *Pti1*-transgenic R0 plants (49-2, 49-4, and 49-7) was probed with the *Pti1* cDNA probe as in Figure 2. The presence of the *Pti1* transgene (1.6 kb) is indicated by the arrowhead.

(B) RNA gel blot of transgenic tobacco plants. RNA was isolated from a wild-type W-38 plant or R0 plants of *Pti1*-transgenic lines 49-2 and 49-4. A 10 μg sample of total RNA was loaded in each lane, and the RNA blot was hybridized to the *Pti1* cDNA probe as in Figure 2.

(C) HR in *Pti1*-transformed tobacco. Fully expanded leaves from 8-week-old plants of tobacco line 49-2 and wild-type tobacco W-38 transformed with pBI121 were inoculated with P. syringae tabaci strain 11528R expressing or lacking *avrPto*. An equal volume (50 μl) of 10^6 cfu/ml bacteria was infiltrated into a region between lateral veins using a syringe. Photographs were taken 13 hr after inoculation.

rapid tissue desiccation. In contrast, W-38 control plants that had been transformed with pBI121 vector alone showed either no HR or a delayed HR 14–16 hr after inoculation. Inoculation of P. syringae tabaci lacking *avrPto* at the same inoculum level did not induce HR on either control or *Pti1*-transformed plants. To verify this finding, we tested R1 progeny of the 49-2 and 49-4 transgenic lines. R1 plants carrying the *Pti1* transgene and progeny plants lacking the *Pti1* transgene were identified by DNA gel blot analysis using the *Pti1* cDNA probe. Three R1 plants from each line that contained the *Pti1* transgene were inoculated with P. syringae tabaci expressing *avrPto*. For controls, three W-38 wild-type plants and two 49-2 *Pti1*-azygous plants were inoculated. Five transgenic plants exhibited the accelerated HR compared with the wild-type

W-38 plants and the 49-2 azygous plants. Thus, the enhanced HR cosegregates with the presence of the *Pti1* transgene.

Discussion

We used the yeast two-hybrid system to identify cDNAs that encode proteins that physically interact with the kinase encoded by *Pto*. One of the interacting clones, *Pti1*, encodes a functional protein kinase with serine/threonine specificity. Pti1 is specifically phosphorylated by Pto, but not by Fen, on serine and threonine residues in an in vitro kinase assay. The interaction requires an invariant lysine residue in subdomain II of Pto, which is also required for autophosphorylation, implying that only phosphorylated Pto is capable of interacting with Pti1. Introduction of a *Pti1* transgene into tobacco plants resulted in an enhanced HR specifically in response to a P. syringae tabaci strain expressing *avrPto*. These results strongly suggest that Pti1 acts as a downstream component in the Pto signaling pathway leading to the disease resistance response.

The recent cloning of several *R* genes provides the first step in elucidating the molecular basis of plant disease resistance controlled by gene-for-gene interactions. However, identification of other components in the various signal transduction pathways and the establishment of their relationships with the *R* gene products are essential for our understanding of disease resistance mechanisms in plants. Despite concerted efforts at mutagenesis, relatively few loci have been identified that are involved in *R* gene–mediated disease resistance. These include *Prf* in tomato (Salmeron et al., 1994), *Nar-1* and *Nar-2* in barley (necessary for powdery mildew resistance mediated by *Mla12*; Freialdenhoven et al., 1994), and *Rcr-1* and *Rcr-2* in tomato (required for leaf mold resistance mediated by *Cf-9*; Hammond-Kosack et al., 1994). One explanation for the paucity of mutants is that the signal transduction pathways specifying disease resistance may involve few components. Alternatively, components in some resistance pathways may be indispensable for plant survival or may occur in multiple copies in the genome. Given the complex nature of the disease resistance response, it is likely there are additional proteins involved that have not yet been revealed by mutagenesis studies. Data presented here demonstrate that the yeast two-hybrid system is a useful alternative for identifying these proteins.

Pti1 Is Involved in the Pto Signaling Pathway

The HR is a major outcome of many incompatible plant–pathogen interactions. A macroscopic HR occurs in bacterial speck–resistant tomato leaves inoculated with high concentrations (10^8 cfu/ml) of P. syringae tomato/*avrPto* and in *Pto*-transgenic tobacco leaves challenged with P. syringae tabaci/*avrPto* (Rommens et al., 1995b; Thilmony et al., 1995). Our results indicate that Pti1 is involved in the Pto-mediated HR. It remains to be shown whether *Pti1* plays a role in other defense responses leading to bacterial speck disease resistance. The availability of transgenic tobacco plants should allow a detailed dissection of the precise function of the *Pti1* gene in the future.

Involvement of a Protein Phosphorylation Cascade in Pto-Mediated Disease Resistance

Protein phosphorylation is the central currency of signal transduction in eukaryotes (reviewed by Hunter, 1995). In plants, protein phosphorylation appears to play a fundamental role in signal transduction pathways involved in several processes, including self-incompatibility, ethylene perception, and defense gene expression (Dietrich et al., 1990; Ecker, 1995; Nasrallah et al., 1994). The involvement of protein phosphorylation in gene-for-gene disease resistance was shown by the discovery that Pto is a serine/threonine kinase (Martin et al., 1993b). The isolation of an additional protein kinase that physically interacts with Pto further supports the central role of a protein phosphorylation cascade in plant disease resistance.

Pti1 Probably Acts Downstream of Pto

The absence of a transmembrane domain or signal peptide sequence in either Pto or Pti1 suggests that these kinases are located in the cytoplasm. In the case of Pto, the putative myristoylation modification at the N-terminus may serve to position this kinase near the cytoplasmic domain of an extracellular receptor (Martin et al., 1993b). In contrast, Pti1 contains no myristoylation site or other features indicating membrane localization. The interaction of Pto with Pti1 requires the kinase activity of Pto, but does not require Pti1 kinase activity. In addition, Pto phosphorylated Pti1 in vitro, but Pti1 was unable to phosphorylate Pto. It is possible that the inability of Pti1 to phosphorylate Pto(K69Q) is due to the lack of a physical interaction between the two proteins. However, in vitro cross-phosphorylation between proteins does not necessarily require their physical interaction. For example, the yeast Ste7 and Ste11 serine/threonine kinases do not interact in the yeast two-hybrid system, but the Ste11 kinase is still capable of phosphorylating Ste7 in vitro (Choi et al., 1994; Neiman and Herskowitz, 1994). A more likely explanation for our results is that Pto is not a substrate of Pti1. Taken together, these observations strongly suggest that Pti1 acts downstream of Pto (Figure 8).

The N-terminal 70 amino acid residues of Pti1 are un-

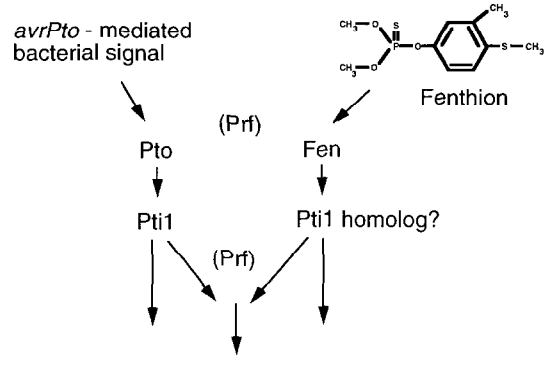

Figure 8. Proposed Model for Pto- and Fen-Mediated Signal Transduction

likely to be required for the kinase activity of Pti1, since they are upstream of the conserved catalytic domain. However, these residues may be involved in the interaction and phosphorylation by Pto. This speculation is based on the observation that threonine residues in Pti1 appear to be the primary targets for phosphorylation by Pto, and five of 11 threonine residues in Pti1 are located within the N-terminal 70 amino acid region. In addition, all three cDNA clones in the *Pti1* class that were isolated via the yeast two-hybrid system contained nearly full-length inserts, indicating that this region may be required for Pto interaction.

Function of Pto and Fen May Involve Different Signal Transduction Components

Our data indicate that, despite the structural similarities between Pto and Fen, only Pto is able to phosphorylate Pti1. This is analogous to many signal transduction pathways in yeast and mammals in which closely related proteins transduce different signals (Neiman, 1993). In the Pto/Fen pathway(s), the biochemical components involved in transducing the signal produced by the avirulent bacterial pathogen and those involved in fenthion sensitivity are different, at least in part, although the two responses may converge. The specific phosphorylation of Pti1 by Pto but not Fen clearly suggests that Pti1 is involved in a function that is mediated by Pto but not Fen. The results presented here suggest that Pti1 does not interact with Fen and that a different component acts downstream of Fen. The fact that *Pti1* belongs to a multigene family raises the possibility that a homolog of Pti1 may serve as a specific substrate for Fen (Figure 8).

A Model for the Pto-Mediated Signaling Pathway

The identification of a Pto-specific substrate supports a model in which Pto and Fen activate independent downstream components in a signal transduction pathway (Figure 8). In this model, the elicitor produced by the avirulent bacterium interacts either directly or indirectly with Pto, and Pto is activated by phosphorylation. Subsequently, phosphorylated Pto recruits and phosphorylates Pti1, resulting in its activation. Ultimately, Pti1 phosphorylates its substrates, leading to the development of HR, which contributes to overall disease resistance.

Although certain components involved in Pto and Fen signaling pathways may be different, four observations suggest that the function of Pto and Fen involves similar mechanisms: phenotypic similarity between fenthion-induced lesions and the HR; a high degree of amino acid sequence similarity between Pto and Fen; overexpression of Pto in transgenic plants confers mild sensitivity to fenthion (Martin et al., 1994); and the *Prf* gene is required for both Pto and Fen function (Salmeron et al., 1994). Prf is not required for resistance to an incompatible strain of Xanthomonas, suggesting that Prf acts relatively early in the signaling pathway (Salmeron et al., 1994). It is possible that Prf acts upstream of Pto and Fen, since it is required for both bacterial speck resistance and fenthion sensitivity (Figure 8). It is also conceivable that Prf functions as a common step downstream of Pto and Fen, likely after Pti1 Isolation of additional proteins that interact with Pto or Pti1 and the cloning of *Prf* should establish the biochemical function of the Prf protein and its role in disease resistance.

The rapid production of H_2O_2 (the oxidative burst) appears to be involved in the HR during incompatible plant–pathogen interactions (Dietrich et al., 1994; Greenberg et al., 1994; Levine et al., 1994). Recently, it was shown that protein phosphorylation is required for the oxidative burst induced in an incompatible interaction between soybean and P. syringae glycinea (Levine et al., 1994). In tomato suspension cells expressing *Pto*, a protein kinase inhibitor prevents the cell death induced by P. syringae tomato carrying *avrPto*, indicating that kinase activity is required for the cell death mediated by Pto (J. Lindell and G. B. M., unpublished data). It remains to be determined whether and how protein phosphorylation actually leads to the HR during incompatible interactions. In human neutrophils, rapid phosphorylation of p47 allows this protein to be relocated to activate the plasma membrane NADPH oxidase and leads to an oxidative burst (Babior, 1992). There is evidence that plants and animals may utilize a similar mechanism for the activation of the oxidative burst (Dwyer et al., 1995; Levine et al., 1994). A possible target for Pti1 could therefore be a tomato homolog of p47.

Pto or Pti1 may also act to regulate gene expression. Transcriptional activation of pathogenesis-related genes and genes involved in phytoalexin biosynthesis, such as chalcone synthase and phenylalanine ammonium-lyase, is common during the disease resistance response (Cutt and Klessig, 1992; Dietrich et al., 1994; Greenberg et al., 1994). It is likely that some of these defense-related genes are also activated in tomato following inoculation with an incompatible P. syringae tomato race and, perhaps, by exposure to fenthion. Phosphorylation of transcription factors is a common mechanism for gene activation in eukaryotes (reviewed by Hunter and Karin, 1992). Recent evidence suggests that protein phosphorylation plays a pivotal role in the regulation of plant defense response genes (Despres et al., 1995; Raz and Fluhr, 1993; Yu et al., 1993). Pto or Pti1 may activate the expression of defense response genes by direct or indirect phosphorylation of transcription factors that are involved in defense gene induction. Interestingly, three Pto-interacting proteins identified in our two-hybrid screen share homology with known transcription factors (J. Z. and G. B. M., unpublished data).

The molecular basis of signal transduction in plants is poorly understood. The identification of the Pti1 kinase and further characterization other proteins that physically interact with Pto provides a model system for study of signal transduction in a plant system in which both the stimulus (the avirulent Pseudomonas bacterium) and the response (disease resistance) are known. The identification of Pti1 substrates and further analysis of the *Pti1*-transgenic plants should establish the precise role of the Pti1 kinase.

Experimental Procedures

The Yeast Two-Hybrid System

The plasmids (pEG202, pJG4-5, pSH18-34, pRFHM-1, and pJK101) and the yeast strain EGY48 (*ura3, his3, trp1, LexAop–LEU2*) were

provided by R. Brent (Massachusetts General Hospital, Boston, MA), and the basic procedures for the yeast two-hybrid system used in this study are as described previously (Golemis et al., 1994; Gyuris et al., 1993; World Wide Web: http://xanadu.mgh.harvard.edu).

The LexA-Pto Construct

To make the in-frame LexA–Pto fusion construct, we PCR amplified the Pto *ORF1* from plasmid CD186 (Martin et al., 1993b) using an EcoRI-tagged upstream primer (5′-GGGAATTCATGGGAAGCAAGTATTC-3′) and a BamHI-tagged downstream primer (5′-CCCTGCAGTGAAAGAAGGATCCACAG-3′), digested it with EcoRI and BamHI, and cloned into the corresponding sites in pEG202. This construct was transformed into the yeast strain EGY48 containing pJK101 to ensure that the LexA–Pto fusion protein is translocated into the nucleus and into EGY48 containing pSH18-34 to test for the possible activation of the reporter genes by the LexA–Pto fusion construct (Golemis et al., 1994).

cDNA Library and Screening of Pto Interactors

We treated 6-week-old plants of Rio Grande–PtoR by dipping them into either 4×10^7 cfu/ml P. syringae tomato T1 (pPtE6) cells (Ronald et al., 1992) or 0.15% fenthion (Martin et al., 1993b, 1994). Equal amounts of leaf tissue were harvested at 0, 4, 8, and 12 hr after treatment, and poly(A)$^+$ RNA was isolated using oligo(dT)–cellulose (Pharmacia, Uppsala, Sweden). The cDNA was synthesized using a cDNA synthesis kit (Stratagene, La Jolla, CA) and ligated into the corresponding sites in pJG4-5. The ligation mixture was transformed into E. coli strain XL2-Blue MRF′ (Stratagene) by electroporation (Sambrook et al., 1989). Approximately 8×10^6 colonies containing the primary cDNAs were obtained.

The plasmid library constructed in pJG4-5 was transformed en masse into the yeast strain EGY48, which contained the LexA–Pto construct and pSH18-34, the *lacZ* reporter plasmid (Golemis et al., 1994). Approximately 2×10^6 transformants grew on glucose lacking uracil, histidine, and tryptophan, and colonies were recovered in Tris–EDTA buffer containing 50% glycerol and stored at −80°C. Approximately 4×10^7 yeast cells were plated on eight 10 cm plates containing galactose agar medium lacking uracil, histidine, tryptophan, and leucine. Large colonies appearing within 3 days were collected and tested on selective media containing X-Gal. pJG4-5 plasmids containing cDNAs were isolated by transforming E. coli strain XL1-Blue (Stratagene) with total genomic DNA prepared from yeast colonies that turned blue on the X-Gal plates (Golemis et al., 1994) and selecting for ampicillin resistance.

To isolate full-length cDNA clones, we used radiolabeled *Pti1* cDNA insert to screen a tomato cDNA library prepared from cultivar OS-4 by plaque hybridization. The library, provided by K. Raghothama, was constructed in the Uni-ZAP XR λ vector (Stratagene).

DNA and RNA Blot Hybridization

DNA gel blot analysis was performed as previously described (Martin et al., 1993a). Hybridization of DNA gel blots and plaque lifts was done at 60°C for 16 hr in 5× SSPE containing 0.1 mg/ml salmon testis DNA, 5× Denhardt's, 0.1% SDS. Filters were washed at 60°C to a stringency of 0.5× SSC containing 0.1% SDS. RNA blots were hybridized with a ^{32}P-labeled cDNA probe in 5× SSPE, 0.1 mg/ml salmon testis DNA, 5× Denhardt's, 0.1% SDS, and 50% formamide at 42°C overnight and washed to 1× SSC containing 0.1% SDS at 60°C.

Assay for β-Galactosidase in Liquid Cultures

A quantitative assay of β-galactosidase activity in liquid culture was performed as described previously (Reynolds and Lundblad, 1989), except that yeast cells were grown in a minimal liquid medium containing 10% raffinose, 10% galactose and lacking uracil, histidine, and tryptophan.

DNA Sequencing and Analysis

The cDNA inserts were excised with EcoRI and XhoI from the pJG4-5 plasmid, subcloned into the corresponding sites in pBluescript SK(−) (Stratagene), and a series of overlapping deletions was generated. Nucleotide sequences were determined using a DNA Sequenase kit version 2.0 (United States Biochemical Corporation, Cleveland, OH). DNA sequence data were analyzed using the GCG software package, version 7.0 (UW Genetics Computer Group, Madison, WI), and the deduced amino acid sequences were compared with sequences in the current databases (GenBank, Swiss Protein, and EMBL) using BLAST, MOTIFS, and BLOCKS.

Expression of the GST-Pti1 Fusion Proteins in E. coli

To fuse the *Pti1* cDNA in-frame to GST, we first digested the pGEX-KG plasmid with EcoRI, repaired the ends with Klenow, and then digested the vector with XhoI. The 1.4 kb *Pti1* cDNA was excised from the pBluescript plasmid with SmaI and XhoI and ligated to the predigested pGEX-KG plasmid. The resulting fusion construct was verified by DNA sequencing and transformed into E. coli strain PR745 (*lon*$^-$; New England Biolabs, Beverly, MA). The GST–Pti1 fusion protein was expressed in E. coli and purified using glutathione affinity chromatography as described previously (Guan and Dixon, 1991). GST was separated from Pti1 protein by cleavage with thrombin.

Kinase Assays and Phosphoamino Analysis

For autophosphorylation assays, 1–3 μg of protein (either in solution or immobilized on 20 μl glutathione agarose beads) was incubated with 10 μCi of [γ-^{32}P]ATP in 50 μl of kinase buffer (50 mM Tris [pH 7.0], 1 mM DTT, 10 mM $MnCl_2$, and 20 mM ATP) at room temperature for 15 min. For cross-phosphorylation assays, 10 μg of MBP–Pto or MBP–Fen fusion protein (Loh and Martin, 1995) was immobilized onto 20 μl amylose beads and incubated with 1–10 μg of GST–Pti1 fusion protein or the cleaved Pti1 protein and 10 μCi of [γ-^{32}P]ATP in 50 μl of kinase buffer for 30 min at room temperature. The reaction was stopped by adding EDTA to a final concentration of 10 mM, and the proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography. The phosphorylated amino acid residues in the ^{32}P-labeled proteins were determined by phosphoamino analysis (Loh and Martin, 1995).

In Vitro Mutagenesis

To make a lysine to asparagine substitution at position 96 in Pti1, single-stranded pBluescript plasmid DNA containing the 1.4 kb *Pti1* cDNA was used as a template. A phosphorylated primer (5′-pGCAGCCATTAATAAATTAGAC-3′) was annealed to the single-stranded template DNA, and the second strand DNA was synthesized using T4 DNA polymerase. The resulting plasmid was ligated and transformed into the E. coli strain XL1-Blue. Colonies containing the desired mutation were identified by digesting the plasmid DNA with VspI, which cuts the mutated but not the wild-type sequence. Plasmid DNA in these colonies consists of half wild-type and half mutant plasmid and was thus retransformed into E. coli until a pure plasmid DNA containing the mutation was isolated.

Tobacco Transformation, Bacterial Strains, and Bacterial Inoculation

The 1.6 kb *Pti1* cDNA was inserted into the binary vector pBI121 (Clontech Laboratories, Palo Alto, CA) between XbaI and SacI sites in the sense orientation. The resulting construct was electroporated into Agrobacterium tumefaciens EHA105 and transformed into Nicotiana tabacum cv. W-38 as described elsewhere (Thilmony et al., 1995). P. syringae tabaci strain 11528R race 0 with or without pPtE6, which contains *avrPto*, was used in inoculation experiments as described (Thilmony et al., 1995), except the plants were kept at 28°C to 30°C.

Acknowledgments

Correspondence should be addressed to G. B. M. We thank Roger Brent for the two-hybrid system, Russ Finley for helpful advice, Kashchandra Ragothama for the tomato cDNA library, Xiaoyan Tang for assistance in construction of the two-hybrid library, Roger Thilmony for help in bacterial inoculation, Dennis Halterman for testing various constructs in the EGY48/Pto strain, Marietta Harrison for use of her thin-layer chromatography apparatus, and Jörg Großhans and Christiane Nüsslein-Volhard for their two-hybrid constructs of Drosophila proteins. For critical reading of the manuscript, we thank Sreeganga Chandra, Reid Frederick, John Hamer, Keith Perry, and members of the Martin laboratory. This research was supported by an F. N. An-

drews predoctoral fellowship (to Y.-T. L.) and National Science Foundation grant MCB-93-03359 (to G. B. M.).

Received June 6, 1995; revised October 19, 1995.

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GenBank Accession Number

The accession number for the sequence reported in this paper is U28007.