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The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes

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In tomato, the Pto kinase confers resistance to bacterial speck disease by recognizing the expression of a corresponding avirulence gene, *avrPto*, in the pathogen *Pseudomonas syringae* pv. *tomato*. Using the yeast two-hybrid system, we have identified three genes, *Pti4*, *Pti5* and *Pti6*, that encode proteins that physically interact with the Pto kinase. *Pti4/5/6* each encode a protein with characteristics that are typical of transcription factors and are similar to the tobacco ethylene-responsive element-binding proteins (EREBPs). Using a gel mobility-shift assay, we demonstrate that, similarly to EREBPs, Pti4/5/6 specifically recognize and bind to a DNA sequence that is present in the promoter region of a large number of genes encoding 'pathogenesis-related' (PR) proteins. Expression of several PR genes and a tobacco *EREBP* gene is specifically enhanced upon *Pto–avrPto* recognition in tobacco. These observations establish a direct connection between a disease resistance gene and the specific activation of plant defense genes.
Keywords: disease resistance/pathogenesis-related protein/protein–protein interactions/tomato/transcription factor

Introduction

It has long been recognized that transcriptional activation of a battery of plant defense-related genes is associated with pathogen invasion (reviewed by Lamb *et al.*, 1989; Bowles, 1990; Dixon and Lamb, 1990). Well documented defense genes include those encoding pathogenesis-related proteins (PRs) (reviewed by Linthorst, 1991; Cutt and Klessig, 1992), hydroxyproline-rich glycoproteins, and enzymes for phytoalexin biosynthesis such as phenylalanine ammonia lyase (PAL) and chalcone synthase (Showalter *et al.*, 1985; Bell *et al.*, 1986). Although the role of these proteins in plant disease resistance has yet to be established, their enzymatic functions indicate that they are well suited for defense against pathogens. For example, two classes of PR proteins, β-1,3-glucanase and chitinase, are lytic enzymes capable of degrading polysaccharides found in the cell wall of many fungi and have antifungal activities when assayed *in vitro* (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993; Melchers *et al.*, 1994; Niderman *et al.*, 1995). Coordinated expression of nine classes of PR gene mRNAs is associated with the onset of systemic acquired resistance (SAR), a general resistance response induced by necrotizing pathogens (Ward *et al.*, 1991). Overexpression of a bean chitinase in tobacco increased resistance to the soil-borne pathogen *Rhizoctonia solani* (Broglie *et al.*, 1991), and overexpression of tobacco PR-1 in tobacco enhanced tolerance to *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.*, 1993). Similarly, overexpression of a tobacco basic PR-5 in potato delayed the disease symptom produced by *Phytophthora infestans* (Liu *et al.*, 1994). These results have spurred extensive investigations into the biological function of defense genes and the mechanisms by which they are activated.

Much effort has been focused on the characterization of *cis*-acting elements involved in elicitor- and pathogen-induced defense gene expression (bean *chs*, Choudhary *et al.*, 1990; parsley *PAL*, Lois *et al.*, 1989; tobacco *PR-1a*, Ohshima *et al.*, 1990; van de Rhee and Bol, 1993; tobacco *PR-2*, Hennig *et al.*, 1993; and *Nicotiana plumbaginifolia gln1*, Alonso *et al.*, 1995). Using these DNA elements as probes, a few putative transcription factors involved in defense responses have been identified. For example, KAP-1 and KAP-2 bind to the H box of the bean chalcone synthase gene *chs15* (Yu *et al.*, 1993), parsley BPF1 binds to box P of the *PAL* gene (da Costa e Silva *et al.*, 1993), parsley and *Arabidopsis* homeodomain proteins bind to the parsley *pr2* gene promoter (Korfhage *et al.*, 1994), and a tobacco 40 kDa DNA-binding protein is thought to be involved in salicylic acid-regulated PR gene expression (Goldsbrough *et al.*, 1993).

Many defense-related genes are induced in both compatible (susceptible) and incompatible (resistant) plant–pathogen interactions. However, the expression of many defense genes is more rapid and pronounced in a resistant plant challenged with an avirulent pathogen. In particular, incompatible interactions involving a plant resistance (*R*) gene and a corresponding pathogen avirulence (*avr*) gene (Flor, 1971) lead to accelerated plant defense gene expression. For example, inoculation of *Arabidopsis* carrying *RPS2* with an avirulent *Pseudomonas* strain containing *avrRpt2* rapidly induced *PAL* mRNA accumulation to a higher level compared with inoculation with a strain lacking the *avr* gene (Dong *et al.*, 1991). Early induction of *ELI3* (elicitor-activated gene) in *Arabidopsis* depends specifically on the expression of the *RPM1* and *avrB* genes in the plant and pathogen, respectively (Kiedrowski *et al.*, 1992). Similarly, *AIG1* and *AIG2* (*avrRpt2*-induced gene) mRNAs are induced in *Arabidopsis* carrying the *RPS2* resistance gene 6 h after infection with *Pseudomonas syringae* pv. *maculicola* containing the corresponding avirulence gene *avrRpt2* but are not induced until 12 h when either *RPS2* or *avrRpt2* is absent (Reuber and Ausubel, 1996). In addition, injection of an intercellular

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fluid containing the Avr9 and Avr2 peptides into tomato plants harboring *Cf-9* or *Cf-2* or of purified Avr9 peptide into *Cf-9* plants induced the expression of two β-1,3-glucanase genes, but not in the plants lacking these *Cf* genes (Ashfield *et al.*, 1994; Wubben *et al.*, 1996).

Many cloned *R* genes encode proteins that are likely to be involved either in the recognition of signals determined by *avr* genes or in the early steps of signal transduction (Martin *et al.*, 1995). However, a direct link between any *R* gene and defense gene activation has yet to be established. The identification of signaling components leading to defense gene activation after *R* gene–*avr* gene recognition would be an important advance. Towards this goal, several *Arabidopsis* mutants have been identified that are either deficient in PR gene expression upon pathogen attack (*npr1*, Cao *et al.*, 1994; *nim1*, Delaney *et al.*, 1995), or constitutively express PR genes (*cpr1*, Bowling *et al.*, 1994). Cloning of these genes and elucidation of the biochemical functions of these gene products will greatly advance our knowledge of plant defense.

In tomato, resistance of plants carrying the *Pto* locus to *Pseudomonas syringae* pv. *tomato* strains expressing the avirulence gene *avrPto* (Ronald *et al.*, 1992; Martin, 1995) is a model system to study signal transduction pathways mediated by a specific *R* gene. This system constitutes the only example of an *R* gene-mediated resistance pathway in which genes for multiple components have been cloned (Martin *et al.*, 1993; Zhou *et al.*, 1995; Salmeron *et al.*, 1996). Currently, three components are known to be involved in the signaling pathway mediated by *Pto*: the serine/threonine protein kinase Pto, a second serine/threonine kinase Pti1 and the leucine-rich repeat type protein Prf. The *Pto* gene was discovered originally in *Lycopersicon pimpinellifolium*, a wild tomato species, and isolated by map-based cloning (Martin *et al.*, 1993). Mutagenesis of a bacterial speck-resistant tomato line revealed a second gene, *Prf* (Salmeron *et al.*, 1994), that is required for both *Pto*-mediated resistance and fenthion sensitivity, a related phenotype mediated by the *Fen* gene (Martin *et al.*, 1994). Recently, we and others have demonstrated that the AvrPto protein, presumably delivered into the plant cell by a type III secretion system of the bacterium, functions directly inside the plant cell (Scofield *et al.*, 1996; Tang *et al.*, 1996). The AvrPto and Pto proteins physically interact and this recognition event initiates the disease resistance response (Scofield *et al.*, 1996; Tang *et al.*, 1996). Using the yeast two-hybrid system with Pto as a bait, we had previously identified another protein kinase Pti1 that appears to act downstream of Pto and is involved in the hypersensitive response (HR; Zhou *et al.*, 1995). Here we report the characterization of three additional Pto-interacting proteins, Pti4, Pti5 and Pti6, hereafter referred to as Pti4/5/6, that belong to a large family of plant transcription factors. These proteins bind to a *cis*-element that is widely conserved among PR genes and are implicated in the regulation of these genes during incompatible plant–pathogen interactions.

Results

Interaction of Pto with Pti4/5/6

Using the yeast two-hybrid system with Pto as a bait, we previously had screened a tomato cDNA library and isolated 149 clones encoding putative Pti proteins (Zhou *et al.*, 1995). These cDNA clones belonged to 10 distinct classes, as indicated by cross-hybridization experiments. The cDNA clones *Pti4*, *Pti5* and *Pti6* represent three of the 10 distinct classes, and were characterized further. Independent clones varying in length that represent identical genes have been identified for each cDNA during the two-hybrid screening (12 for *Pti4*, nine for *Pti5* and one for *Pti6*). Figure 1 shows the specific interaction of Pti4, Pti5 and Pti6 with Pto in yeast. Yeast strains carrying the Pto bait and a prey of Pti4, Pti5 or Pti6 grew in the absence of leucine, indicative of the *LEU2* reporter gene activation (data not shown). When grown on X-Gal plates, these yeast cells were blue as a result of the *lacZ* reporter gene activation. As determined by the intensity of blue color, the strength of interaction of Pto with these three preys is in the order Pti6>Pti4>Pti5. In contrast, control yeast strains expressing the arbitrary bait Bicoid and any one of the three preys did not activate the *LEU2* or the *lacZ* reporter genes. Previously, we have cloned the recessive *pto* allele from a tomato cultivar that is susceptible to *P.s.tomato* carrying *avrPto* (Jia *et al.*, 1997). The amino acid sequence of pto is 87% identical to Pto. However, the pto bait did not interact with Pti4, Pti5 or Pti6 in yeast as shown by the lack of *lacZ* activation (Figure 1). The slight blue color of colonies expressing pto and Pti6 developed only after prolonged incubation and probably does not represent significant physical interaction of these proteins. In addition, other kinases such as Pti1 and Fen as baits did not interact with Pti4/5/6 (data not shown). These results indicate that the interactions of these Pti proteins with Pto were highly specific.

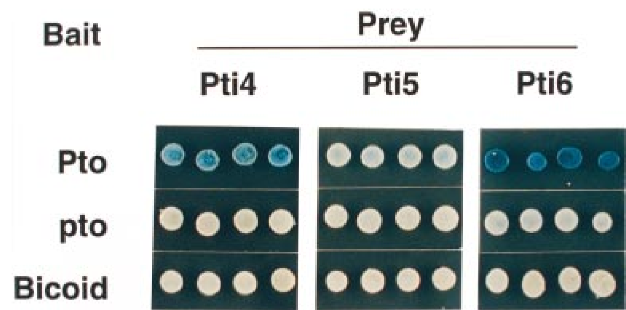


Fig. 1. Interaction of Pto with Pti4/5/6. EGY48 yeast cells containing a prey of Pti4, Pti5 or Pti6 (in pJG4-5), and a bait of Pto, pto or Bicoid (in pEG202) were grown on galactose Ura^- His^- Trp^- X-Gal medium. The plates were incubated at 30°C for 3 days and photographed. Four independent, representative colonies are shown for each bait–prey combination. All bait proteins were expressed equally in yeast as determined by Western blots (Y.-T.Loh and G.B.Martin, unpublished results).

Pti4/5/6 are members of a multigene family

The longest *Pti4/5/6* cDNAs isolated from the two-hybrid screen were 1.0, 0.8 and 0.8 kb, respectively. Each of these cDNAs was probed onto genomic blots containing DNA from tomato cultivar Rio Grande-PtoR (Figure 2A). Numerous fragments were detected by both *Pti4* and *Pti5* cDNA probes, while 2–3 fragments were detected by the *Pti6* cDNA probe. The distinct restriction fragments detected by these probes indicated that the sequences of *Pti4/5/6* are not identical. However, a cross-hybridization experiment indicated weak homology among the three

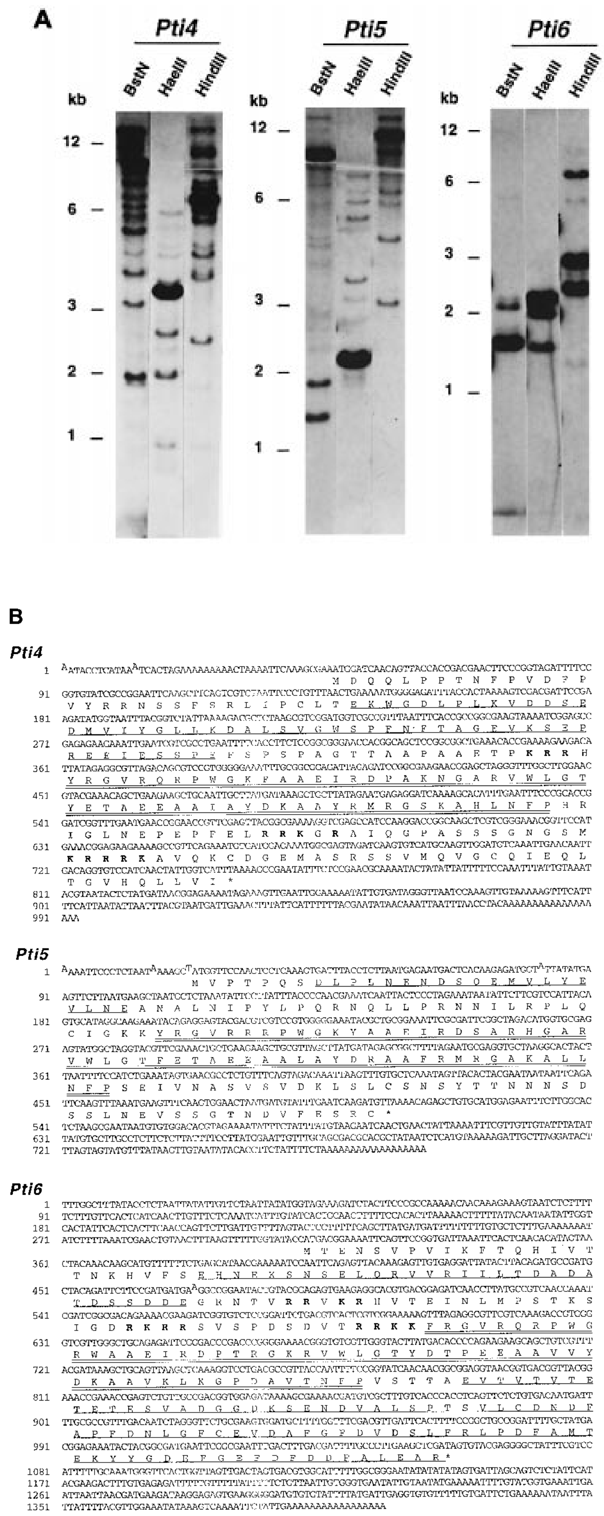


Fig. 2. Pti4/5/6 belong to a large gene family. (**A**) DNA gel blot analysis of tomato genomic DNA. Genomic DNA (5 µg/lane) from Rio Grande-PtoR plants was digested with the indicated restriction enzymes, and the DNA blot was hybridized to the *Pti4/5/6* cDNA probes. (**B**) Nucleotide and deduced amino acid sequences of Pti4/5/6. Superscript letters denote the 5′ ends of cDNA clones recovered from the two-hybrid library screening. Amino acids with a single underline are acidic regions, whereas those with a double underline represent the conserved central basic region. Putative nuclear localization sequences are shown in bold. Nucleotide sequence data have been deposited in DDBJ/EMBL/GenBank (Pti4, accession No. U89255; Pti5, U89256; Pti6, U89257).

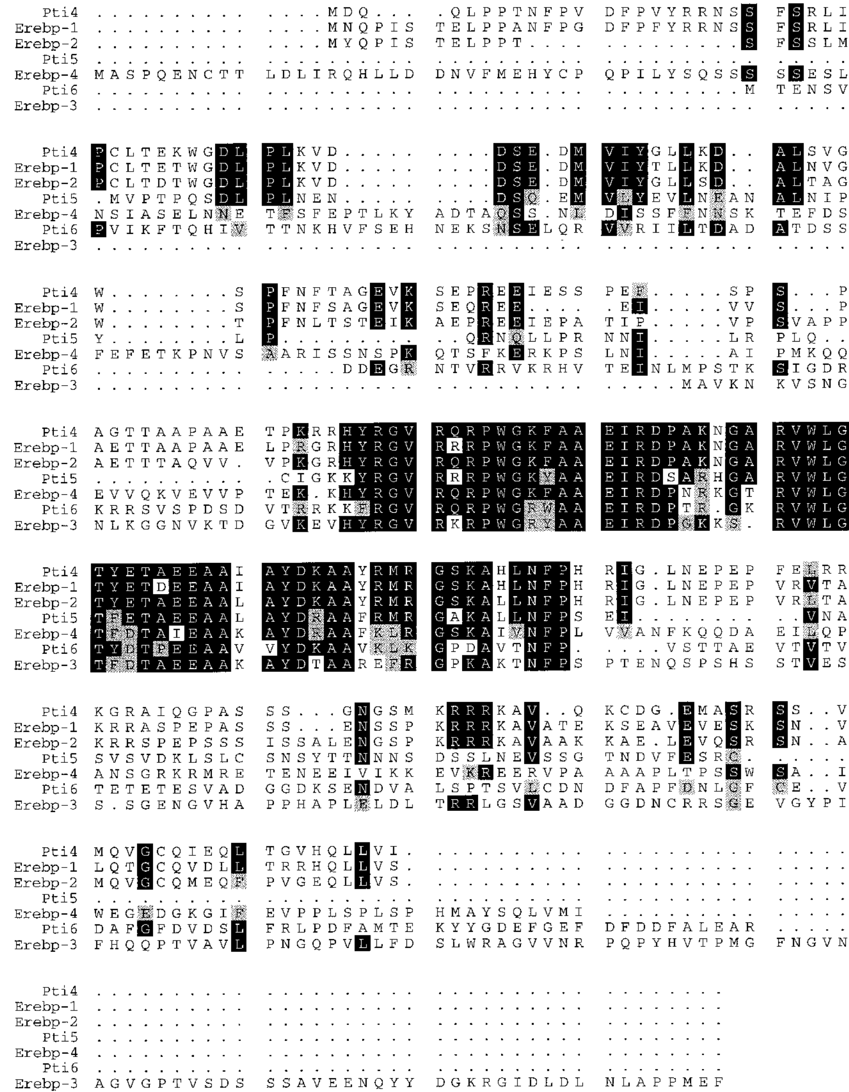
classes of cDNAs (data not shown). Subsequent DNA sequence analysis confirmed this homology among Pti4/5/6 proteins (see below). Thus *Pti4/5/6* appear to be members of a large gene family.

We sequenced the inserts of *Pti4/5/6* and found that each contained a single open reading frame that was fused in-frame to the activation domain of the prey plasmid (Figure 2B). To isolate full-length cDNA clones, we screened another tomato cDNA library with the *Pti4/5/6* cDNA probes. This screen resulted in the isolation of longer cDNA clones for *Pti6* but not for *Pti4* and *Pti5*. The longest *Pti6* cDNA clone is 1.4 kb and corresponds to the transcript size detected by a Northern blot, indicating that this *Pti6* clone is probably full-length (data not shown). The original *Pti4* and *Pti5* clones are also likely to be full-length as they both contain a putative start codon at their 5′ ends (see below).

The putative proteins encoded by *Pti4/5/6* contain 234, 161 and 248 amino acid residues, respectively (Figure 2B). The Pti6 protein expressed from the original prey plasmid lacked the first 48 amino acid residues, indicating that these residues are not essential for interaction with Pto. Several features that are typical of transcription factors are present in the Pti4/5/6 proteins. Most strikingly, a central domain of 58 amino acids rich in basic residues is shared by all three proteins (62–81% identical), and is reminiscent of the DNA-binding domain of many transcription factors (Latchman, 1995). Pti4 and Pti6 contain short clusters of basic residues similar to known nuclear localization sequences (NLS; reviewed by Dingwall and Laskey, 1991). In addition, Pti4 and Pti5 each contain an acidic region N-terminal to the central region, and Pti6 contains acidic regions both N- and C-terminal to the central domain. Many transcription factors, such as the yeast GAL4 protein, the mammalian glucocorticoid receptor and the maize Viviporous-1 protein, contain acidic regions that function in transcriptional activation (McCarty *et al.*, 1991; Latchman, 1995). When Pti5 and Pti6 were expressed independently as baits in the yeast strain EGY48, they showed strong autoactivation of the reporter genes in the absence of any prey (data not shown). This suggests that at least Pti5 and Pti6 contain functional transactivation domains.

Pti4/5/6 bind the PR box that is conserved among many pathogenesis-related genes

Using the BLAST program, we searched the DDBJ/EMBL/GenBank database (version 86.0) for proteins with similarity to Pti4/5/6. All protein sequences retrieved from the database shared significant homology with the basic, central domain of Pti4/5/6. These proteins include, in the order of similarity to Pti4/5/6, the tobacco ethylene-responsive element-binding proteins (EREBPs; Ohme-Takagi and Shinshi, 1995), and the *Arabidopsis* proteins TINY (Wilson *et al.*, 1996), AP2 (Jofuku *et al.*, 1994) and ANT (Elliott *et al.*, 1996; Klucher *et al.*, 1996). A number of expressed sequence tag (EST) sequences of unknown function from both *Arabidopsis* and rice also contain this central domain. EREBPs contain a single central domain that has been demonstrated recently to bind a *cis*-acting element conferring the ethylene responsiveness of the β-1,3-glucanase gene *gln2* in tobacco (Ohme-Takagi and Shinshi, 1995). This ethylene-



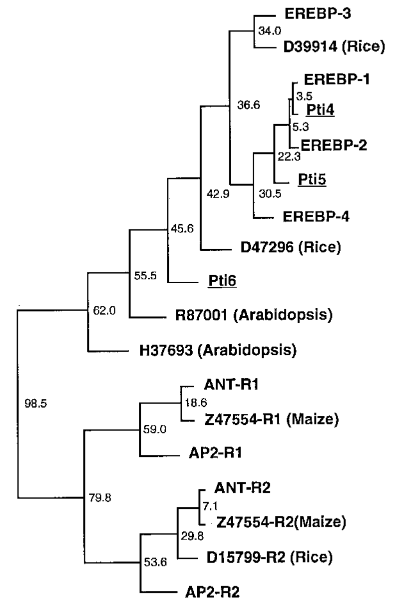


Fig. 3. Similarity of Pti4/5/6 to EREBP/AP2 transcription factors. (**A**) Alignment of Pti4/5/6 amino acid sequence with EREBPs from tobacco. The Pretty Box program (GCG package, version 7.0) was used to create the best alignment. Amino acids identical in at least four of the sequences are shaded in black and conservative substitutions are shaded in gray. (**B**) Phylogenetic tree of transcription factors in the EREBP/AP2 family. The putative DNA-binding region was used to create the best alignment with the Pileup program (GCG package, version 7.0), and the phylogram was created using the Neighbor-Joining method of the Growtree program (GCG package, version 7.0). The length of branches indicates the relative evolutionary distances. Sequences used in this analysis include the single DNA-binding region of Pti4/5/6, four tobacco EREBPs (Ohme-Takagi and Shinshi, 1995), two *Arabidopsis* EST clones (accession Nos R87001 and H37693), two rice EST clones (accession Nos D39914 and D47296), the two DNA-binding regions in *Arabidopsis* proteins AP2 (Jofuku *et al.*, 1994) and ANT (Elliott *et al.*, 1996; Klucher *et al.*, 1996), the maize protein Zmmhcf1 (accesssion No. Z47554) and the second DNA-binding region of a rice EST clone (truncated at its first DNA-binding domain, accession No. D15799).

responsive element contains the core sequence GCCGCC. Since this sequence has been found in the promoters of many PR genes (see below), we refer to it as the PR box. *TINY* is an *Arabidopsis* gene encoding a protein with a single central domain and is involved in determining plant height, hypocotyl elongation and fertility (Wilson *et al.*, 1996). AP2 and ANT are involved in flower development, and both contain two central domains that are also believed to bind DNA (Jofuku *et al.*, 1994; Elliott *et al.*, 1996; Klucher *et al.*, 1996).

Figure 3A shows an alignment of the amino acid sequences of Pti4/5/6 and the EREBPs. The homology among these sequences resides primarily in the DNA-binding domain (at least 67% identical). However, Pti4, EREBP-1 and EREBP-2 are highly similar (71–78% identity) to each other throughout their entire open reading frames. The N-terminus of Pti5 also shares significant homology with Pti4, EREBP-1 and EREBP-2, and the entire Pti5 protein is 43–48% identical to these three proteins. In contrast, Pti6, EREBP-3 and EREBP-4 are more distantly related, and their sequences outside the DNA-binding domain share little homology with any known proteins.

To explore further the relationships of the proteins containing the EREBP DNA-binding domain, evolutionary distances among these sequences were calculated and a phylogenetic tree was constructed (Figure 3B). The sequences clearly divided into two major groups, with

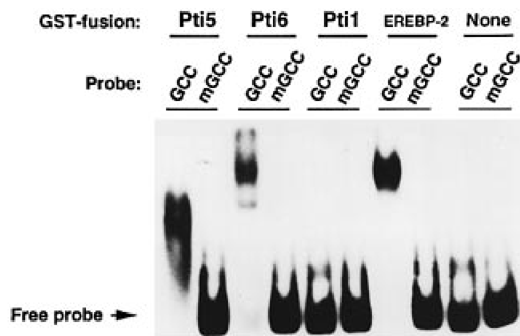


Fig. 4. Pti5 and Pti6 bind the PR box. Fifty ng of purified GST fusion proteins were mixed with the wild-type PR box probe (GCC) or the mutated PR box probe (mGCC), and the gel mobility-shift assay was performed as described in Materials and methods.

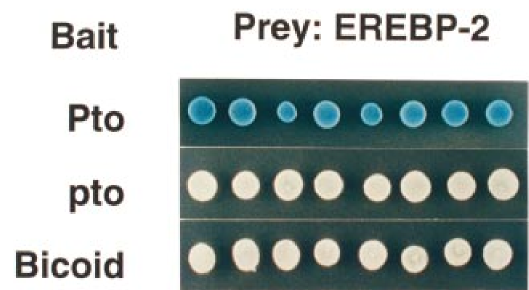


Fig. 5. Interaction of EREBP-2 with Pto. EGY48 yeast cells containing the EREBP-2 prey and a bait of either Pto, pto or Bicoid (in pEG202) were grown on galactose Ura^- His^- Trp^- X-Gal medium. The plates were incubated at 30°C for 3 days and photographed. Eight independent, representative colonies are shown for each bait–prey combination.

one consisting of proteins with a single DNA-binding domain, and the other of proteins with two DNA-binding domains. Pti4/5/6, EREBPs and several other EST sequences belong to the first group, whereas the proteins involved in flower development are in the second group. Thus, Pti4/5/6 are most closely related to EREBPs and are more distantly related to floral development regulators such as AP2 and ANT.

It has been shown previously that the central, basic domain of the EREBPs binds the wild-type PR box of *gln2* but not a mutated PR box harboring two G→T substitutions in the GCCGCC core sequence (Ohme-Takagi and Shinshi, 1995). The similarity of the Pti4/5/6 proteins and the EREBPs led us to hypothesize that Pti4/5/6 also bind to the PR box. To test this possibility, we expressed the full-length Pti6 and the C-terminal 141 amino acids of Pti5 as GST fusion proteins. For a positive control, we expressed the tobacco EREBP-2 protein as a GST fusion. The ability of GST–Pti5 and GST–Pti6 to bind the wild-type or the mutated *gln2* PR boxes was tested using a mobility-shift gel assay. As shown in Figure 4, both GST–Pti5 and GST–Pti6 bound the wild-type PR box similarly to GST–EREBP-2. No binding was detected when the mutated PR box was used in the assay, indicating that binding of GST–Pti5 and GST–Pti6 to the PR box was highly specific. In contrast to GST–Pti5 and GST–Pti6, neither GST–Pti1 nor GST itself bound to the PR box. These results further confirmed the specificity of binding of Pti5 and Pti6 to the *gln2* PR box. Thus the DNA-binding domain of both Pti5 and Pti6 is functionally homologous to that of EREBPs. We have been unable to express Pti4 in *Escherichia coli* and were therefore unable to test its activity in this assay. However, given the high degree of homology between Pti4 and EREBP-2, Pti4 is also likely to bind to the PR box.

Interaction of Pto with tobacco EREBP-2

The sequence similarity and PR box-binding properties of Pti/4/5/6 and the tobacco EREBPs strongly suggest that these proteins are functionally homologous. Introduction of the tomato *Pto* gene into tobacco is known to enhance defense responses specifically upon inoculation with *P.s.tabaci* expressing *avrPto* (Thilmony *et al.*, 1995). In addition, tobacco cultivar W-38 contains a functional, endogenous *Pto* gene that also specifically recognizes the expression of *avrPto* in *P.s.tabaci* (Thilmony *et al.*, 1995). Together these observations imply that the *Pto*-mediated signaling pathway is conserved in both tomato and tobacco and that the tomato Pto protein is compatible with other signaling components from tobacco. We thus predicted that at least some of the tobacco EREBPs would interact with the tomato Pto protein.

The high degree of homology shared between EREBP-2 and Pti4 suggests that EREBP-2 is a functional homolog of Pti4. We therefore tested the ability of EREBP-2 to interact with Pto. Expression of EREBP-2 as a prey in yeast strain EGY48 carrying the tomato Pto bait strongly activated both the *lacZ* and the *LEU2* reporter genes, indicating interaction of Pto with EREBP-2 (Figure 5; J.Zhou and G.B.Martin, unpublished results). This interaction is highly specific, as the pto bait and the Bicoid bait failed to interact with EREBP-2. This experiment thus supports our hypothesis that the tobacco EREBP-2 protein is a functional homolog of Pti4/5/6.

Activation of PR gene expression upon Pto–avrPto recognition

Our results suggest that Pti4/5/6 and EREBPs are transcription factors involved in the regulation of gene expression by binding to the PR box. A search for plant promoter sequences containing the PR box core sequence (GCCGCC) uncovered a number of predominantly basic PR genes from bean, tobacco, potato, *Arabidopsis* and tomato (Table I). It is thus plausible that Pto regulates PR gene expression in tomato via interaction with PR box-binding proteins such as Pti4/5/6 and EREBPs.

Next we examined whether there was a correlation between *avrPto–Pto* recognition and induction of PR genes containing the PR box. We chose tobacco to address this question because PR gene expression is easily monitored in this species, and a number of PR gene promoters have been analyzed in detail in tobacco. In addition, the Pto-mediated signal transduction pathway is conserved between tomato and tobacco (Rommens *et al.*, 1995; Thilmony *et al.*, 1995).

Tobacco leaves (W-38) either with or without the tomato *Pto* transgene were injected with *P.s.tabaci* strains with or without *avrPto* (at a level of 10^6 c.f.u./ml). We examined the mRNA levels of three PR genes that contain PR boxes in their promoter: the *PRP1* gene encoding a basic PR-1 protein (Payne *et al.*, 1989), the basic chitinase gene *CHN50* (Fukuda *et al.*, 1991) and the *Osmotin* gene

Table I. Occurrence of PR-box in plant defense genes

Plant	Gene name	Protein encoded	Reference
N.plumbaginifolia	*gn1*	β-1, 3-glucanase	Castresana *et al.* (1990)
N.plumbaginifolia	*gn2*	basic β-1,3-glucanase	Gheysen *et al.* (1990)
N.tabacum	*CHN50*	basic chitinase	Fukuda *et al.* (1991)
N.tabacum	*CHN17*	basic chitinase	Shinshi *et al.* (1990)
N.tabacum	*CHN14*	basic chitinase	van Buuren *et al.* (1992)
N.tabacum	*GLA (gln2)*	basic β-1,3-glucanase	Sperisen *et al.* (1991) and Ohme-Takagi and Shinshi (1990)
N.tabacum	*GLB*	basic β-1,3-glucanase	Sperisen *et al.* (1991)
N.tabacum	*PRP1*	basic PR-1	Payne *et al.* (1989)
N.tabacum	*prb-1b*	basic PR-1	Meller *et al.* (1993)
N.tabacum	*Osmotin*	basic PR-5	Liu *et al.* (1995)
N.tabacum	*OPL*	neutral PR-5	Sato *et al.* (1996)
N.tabacum	*chi-v*	class V chitinase	Melchers *et al.* (1994)
S.tuberosum	*WIN2*	wound inducible (PR-4-like)	Stanford *et al.* (1988)
S.tuberosum	*STPRINPSG*	protease inhibitor	Y.Choi *et al.* (DDBJ/EMBL/GenBank Z12824)
S.commersonii	*pOSML13*	basic PR-5	Zhu *et al.* (1995)
S.commersonii	*pOSML81*	basic PR-5	Zhu *et al.* (1995)
L.esculentum	*CHN*	basic chitinase	cited by Hart *et al.* (1993)
P.vulgaris	*CH5B*	basic chitinase	Broglie *et al.* (1989)
A.thaliana	*CHA2*	basic chitinase	Samac *et al.* (1990)
A.thaliana	*PAL3*	phenylalanine ammonia-lyase	Wanner *et al.* (1995)
B.napus	*Bp10*	ascorbate oxidase	Albani *et al.* (1992)

encoding a basic PR-5 protein (Liu *et al.*, 1995). Figure 6 shows that transcripts of the three genes accumulated after inoculation with *Pseudomonas* bacteria. In the non-transgenic plants, inoculation of *P.s.tabaci* containing *avrPto* induced the three transcripts at 18 h. In contrast, injection of *P.s.tabaci* without the *avrPto* gene in the same plants delayed transcript accumulation. The early induction of PR genes by avirulent bacteria in the non-transgenic plants is likely to be a result of the recognition of *avrPto* by the endogenous *Pto* homolog in this particular tobacco cultivar (Thilmony *et al.*, 1995). In the *Pto* transgenic plants, this early induction by *P.s.tabaci* carrying *avrPto* was accelerated further, with the earliest expression observed at 9 h after inoculation. Surprisingly, the induction of the PR genes in the *Pto* transgenic plants by virulent bacteria (*P.s.tabaci* without *avrPto*) was also accelerated and occurred at 18 h after infection. One possible explanation for this induction is that an unknown *avr* gene is present in the *P.s.tabaci* strain and is recognized by the tomato *Pto* gene. Alternatively, overexpression of the tomato *Pto* gene in tobacco may potentiate PR gene induction by *Pseudomonas* bacteria. Together, these results support a role for *Pto* in rapid expression of PR genes containing the PR box.

To test further the functional relevance of EREBPs in resistance responses, we examined mRNA accumulation of *EREBP-1* and *EREBP-2*, which have the highest homology to *Pti4* and *Pti5*. We were unable to detect reliably the expression of *EREBP-2* mRNA. However, we found that *EREBP-1* mRNA was induced in the *Pto* transgenic tobacco plants at a high level 9 h after infection with *P.s.tabaci* expressing *avrPto* (at an inoculum level of 10^6 c.f.u./ml; Figure 7A). In contrast, *EREBP-1* mRNA did not accumulate until 18 h after inoculation with *P.s.tabaci* lacking *avrPto*. Similar results were observed with non-transgenic tobacco plants, suggesting that the endogenous *Pto* homolog is sufficient for the *EREBP-1* activation. These experiments indicated that *EREBP-1* mRNA accumulated earlier than that of the PR mRNAs during incompatible interactions. To investigate this observation

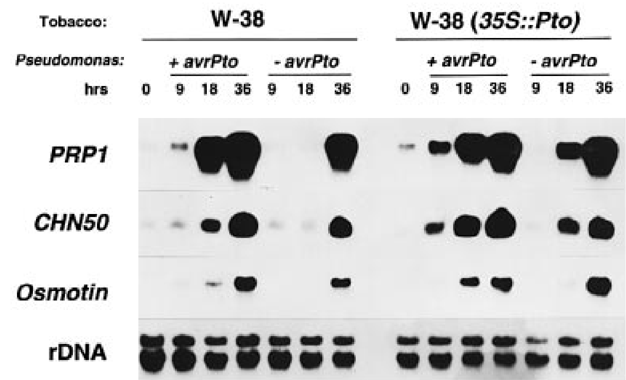


Fig. 6. Induction of tobacco PR gene expression upon *Pto–avrPto* recognition. Tobacco leaves, either non-transgenic (W-38) or with the *Pto* transgene (*35S::Pto*), were injected with *P.s.tabaci* at 10^6 c.f.u./ml. Leaf tissue was harvested at the indicated times post-inoculation, and RNA was extracted. Duplicates of RNA gel blots were hybridized to tobacco *PRP1*, *CHN50*, *Osmotin* or to rDNA probes. The experiment was repeated three times with the same results.

further, we inoculated the *Pto* transgenic tobacco leaves with high inocula (10^8 c.f.u./ml) of *P.s.tabaci* with or without *avrPto* and examined the expression of *CHN50*, *Osmotin* and the *EREBP-1* mRNAs. Figure 7B shows that the presence of *avrPto* in *P.s.tabaci* and the use of high inocula further accelerated the expression of these genes. The avirulent bacteria (with *avrPto*) induced the expression of *CHN50* and the basic *PR-5* at 6 h post inoculation, and that of *EREBP-1* within 3 h after inoculation. The virulent bacteria (no *avrPto*) at high inoculum (10^8 c.f.u./ml) did not induce *EREBP-1* mRNA accumulation within the 9 h time frame of the experiment. However, the virulent bacteria (10^8 c.f.u./ml) induced *CHN50* and *Osmotin* at 9 h post-inoculation, indicating that *EREBP-1* expression is not required for the induction of these PR genes by virulent bacteria. Thus, the early induction of *EREBP-1* mRNA correlates with the subsequent PR gene induction only in the incompatible interactions but not in the compatible interactions. It is conceivable that the avirulent

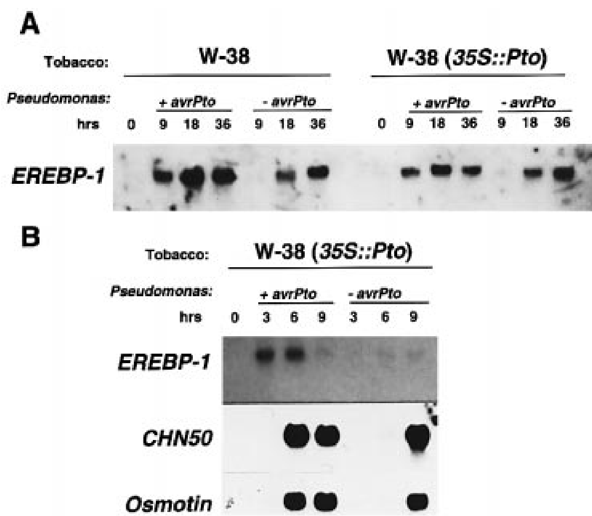


Fig. 7. Induction of tobacco *EREBP-1* expression upon *Pto–avrPto* recognition. Tobacco leaves, either non-transgenic (W-38) or with the *Pto* transgene (*35S::Pto*), were injected with *P.s.tabaci* at 10^6 c.f.u./ml (**A**) or at 10^8 c.f.u./ml (**B**). Leaf tissue was harvested at the indicated times post-inoculation, and RNA was extracted. Duplicates of RNA gel blots were hybridized to the tobacco *CHN50*, *Osmotin* or *EREBP-1* probes.

and virulent bacteria activate defense gene expression through different signal transduction pathways (Dong *et al.*, 1991).

Discussion

Interaction of the Pto kinase with transcription factors

Using the yeast two-hybrid system, we identified three distinct genes encoding putative transcription factors that interact specifically with the Pto kinase. We assessed the significance of this finding first by testing the specificity of the interactions. While Pto interacted with Pti4/5/6, interactions were not observed with any other tested bait proteins. In particular, the lack of interaction of pto with Pti4/5/6 is consistent with the fact that tomato plants carrying *pto* are susceptible to *P.s.tomato* (*avrPto*). Pti4/5/6 all contain a highly conserved DNA-binding domain, and this domain is also involved in the interaction with Pto (data not shown). *Arabidopsis* transcription factors such as MADS proteins also require the DNA-binding domain for certain protein–protein interactions (Mizukami *et al.*, 1996). Secondly, we demonstrated that Pti4/5/6 are functionally similar to EREBPs in both structure and DNA-binding properties, suggesting that Pti4/5/6 are involved in defense gene regulation. This possibility is strengthened further by the strong interaction between EREBP-2 and Pto. The relevance of a PR box-binding factor to *R* gene-determined defense responses is also supported by the fact that *EREBP-1* is induced early upon *Pto–avrPto* recognition, and the induction of *EREBP-1* precedes that of basic *PR-5* and *PRP1* in the incompatible interactions. This is similar to the induction of the parsley *PAL* gene in response to an elicitor derived from *Phytophthora megasperma* f. sp. *glycinea* (da Costa e Silva *et al.*, 1993). Accumulation of *BPF-1* mRNA that encodes a putative transcription factor precedes *PAL* mRNA expression. Taken together, these findings suggest that Pti4/5/6 and at least some of the EREBPs are probable components linking an *R* gene product (Pto) to the expression of specific defense-related genes.

The significance of these findings are 2-fold. Transcriptional regulation of defense genes is thought to be important for the establishment of plant disease resistance (Lamb *et al.*, 1989). Several plant transcription factors are thought to play a role in defense gene regulation (da Costa e Silva *et al.*, 1993; Yu *et al.*, 1993; Korfhage *et al.*, 1994). However, none of them have been shown to be associated with an *R* gene-specified resistance pathway. The identification of Pti4/5/6 as putative transcription factors linking PR gene expression to an *R* gene product therefore promises new insight into how defense gene expression, one of the best known defense responses, is activated after *R* gene–*avr* gene recognition. Secondly, although regulation of transcription factors by protein kinases is a common mechanism in eukaryotes (Hunter and Karin, 1992), relatively little is known of this mechanism in plants. Phosphorylation of the *Arabidopsis* transcription factor GBF1 by casein kinase II was shown to enhance the binding of GBF1 to the G box *cis*-element (Klimczak *et al.*, 1992, 1995), whereas dephosphorylation by a phosphatase reduced the binding (Harter *et al.*, 1994). Likewise, phosphorylation of the potato transcription factor PBF-1 is believed to play a role in *PR-10a* expression (Despres *et al.*, 1995). However, specific protein kinases that are involved in regulation of these transcription factors have yet to be identified. Further investigation of the interaction of the Pti4/5/6 with the Pto kinase should help to elucidate the potential role of protein phosphorylation in the transcriptional regulation of plant defense genes.

Regulation of PR gene expression in disease resistance

PR gene expression is known to be activated by a number of biotic or abiotic stresses including infection by viral, bacterial or fungal pathogens, treatment with plant hormones and elicitors such as ethylene, salicylic acid (SA), jasmonic acid, H_2O_2 and UV light (reviewed by Bol *et al.*, 1990; Linthorst, 1991), and necrotic lesions induced by either mutations or ectopic expression of foreign genes (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Mittler *et al.*, 1995; Herbers *et al.*, 1996). Whereas expression of PR genes is generally induced by pathogen infection, their expression is not always enhanced by *R* gene–*avr* gene recognition (Danhash *et al.*, 1993). Here we demonstrated that expression of *PRP1*, *CHN50*, *Osmotin* and *EREBP-1* in tobacco are enhanced specifically by the recognition of *Pto–avrPto* genes. These findings are consistent with the proposed role of EREBPs in the Pto-mediated signaling pathway.

It is not clear whether Pti4/5/6 are functionally redundant. The sequence divergence outside the DNA-binding domains suggests a functional difference among Pti4/5/6. One possibility is that Pti4/5/6 each has a preference for a distinct PR gene promoter. For example, the context surrounding the GCCGCC core sequence in various PR genes may influence binding affinities of the Pti4/5/6 proteins. Indeed, we have observed that EREBP-2, Pti5 and Pti6 bind to the PR box of *gln2* better than to that of the basic *PR-5* gene (J.Zhou and G.B.Martin, unpublished results). The possibility that different defense genes are

regulated by these transcription factors is consistent with the observation that a diverse array of defense genes are activated in the resistance response. Further examination of the role of each of these transcription factors in the Pto signaling pathway will enable us to address this question.

Although PR gene expression is known to inhibit fungal growth in plants, whether PR proteins have antibacterial activity remains uncertain. However, existing evidence indicates that PR proteins are also likely to be involved in the bacterial disease resistance. For example, the *Arabidopsis* mutant *npr1* is unable to express PR genes and fails to restrict the growth of the bacterial pathogen *P.s.maculicola* (Cao *et al.*, 1994). Conversely, the *Arabidopsis* mutant *cpr1*, that constitutively expresses PR genes, shows heightened resistance not only to the fungal pathogen *Peronospora parasitica* but also to the bacterial pathogen *P.s.maculicola* (Bowling *et al.*, 1994). Thus, the increased PR gene expression upon *Pto–avrPto* recognition may contribute to the overall resistance to the *Pseudomonas* bacteria.

One strategy to improve disease resistance in crop plants is by overexpressing antimicrobial proteins such as PR proteins. However, manipulation of individual PR genes has only led to a partial tolerance to several fungal pathogens (Broglie *et al.*, 1991; Alexander *et al.*, 1993; Liu *et al.*, 1994). Recent studies by Zhu *et al.* (1994) and Jach *et al.* (1995) demonstrated that co-expression of chitinase and glucanase genes in tobacco resulted in a synergistic enhancement of resistance to *Cercospora nicotianae* and *Rhizoctonia solani*, respectively. Similarly, simultaneous overexpression of the class I chitinase and the class I glucanase genes in tomato synergistically enhanced resistance against the *Fusarium* wilt disease (Jongedijk *et al.*, 1995). In systemic acquired resistance, a large number of PR genes are coordinately expressed, and this type of resistance is known to be effective against a wide range of pathogens (Ward *et al.*, 1991). A more effective approach to engineer crop plants with enhanced protection against pathogens might be to coordinately express a large combination of PR proteins in the plant. However, transferring multiple genes into a plant is technically difficult. The discovery of Pti4/5/6 and EREBPs as PR box-binding factors may provide a new approach to improving crop plants with enhanced PR gene expression and disease resistance.

Cross-talk between the ethylene and disease resistance pathways

It has long been known that overlapping sets of genes are induced by both ethylene and pathogen infection (Boller and Vogeli, 1984; Ecker and Davis, 1987; Mauch and Staehelin, 1989). This has led to the hypothesis that ethylene may be a component in the signaling pathway of plant defense responses (Raz and Fluhr, 1992). However, genetic studies with ethylene-insensitive mutants clearly indicate that ethylene action is not required for disease resistance responses in *Arabidopsis* (Bent *et al.*, 1992; Lawton *et al.*, 1994). Although ethylene is unlikely to be a component in the signaling chain leading to disease resistance, it is conceivable that the disease resistance pathways and the ethylene pathway converge at transcription factors such as Pti4/5/6 and EREBPs. In addition to serving as an ethylene-responsive element (Hart *et al.*, 1993; Sessa *et al.*, 1995; Ohme-Takagi and Shinshi, 1995), the PR box may also be involved in the pathogen-activated transcription of PR genes (Alonso *et al.*, 1995). It is a common observation that certain transcription factors serve as targets for different signaling pathways. For instance, the extensively studied animal transcription factor CREB (cAMP response element-binding protein) is phosphorylated and activated by protein kinases from a number of different signaling pathways (reviewed by Hill and Treisman, 1995). It will be interesting to determine whether Pti4/5/6 and EREBPs are regulated both by protein kinases, such as Pto, that function specifically in disease resistance and by a MAP kinase that functions downstream of the CTR kinase in the ethylene pathway (Kieber *et al.*, 1993).

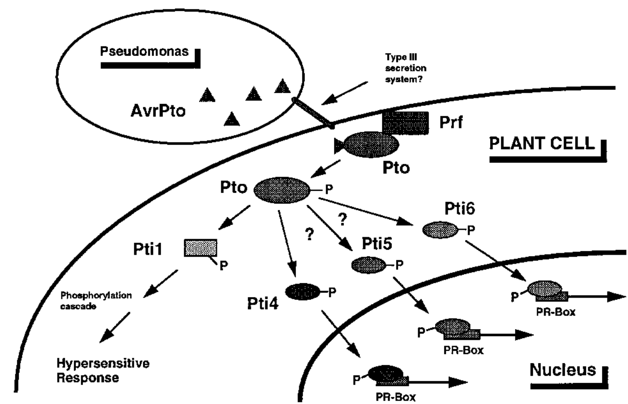


Fig. 8. Proposed model for Pto-mediated signal transduction.

A model for the Pto signaling pathway

Two of the major outcomes of *R* gene-mediated signaling pathways are the hypersensitive response (HR) and defense gene expression. Several reports have suggested that the HR and defense gene expression result from distinct pathways. For example, *Pseudomonas* harboring mutations in *hrp* genes (required for the hypersensitive response in non-host and pathogenicity in host plants) fail to elicit an HR but still induce defense gene expression in bean (Jakobek and Lindgren, 1993). The *rps2-201* mutant is unable to develop an HR in response to *P.s.tomato* carrying *avrRpt2* but still develops systemic acquired resistance (Cameron *et al.*, 1994). Furthermore, the *Arabidopsis* mutant *npr1*, in which SA- and pathogen-induced PR gene induction are abolished, has an intact HR in response to *P.s.maculicola* (*avrRpt2*) (Cao *et al.*, 1994). These observations are consistent with the notion that the signal perceived by an *R* gene product is transduced via two separate pathways, namely an HR pathway and a defense gene activation pathway, although cross-talk may exist between the two pathways.

During *Pto*-mediated resistance responses, the pathways leading to HR and defense gene activation are likely to bifurcate after Pto. Figure 8 depicts our current model for the Pto signaling pathway. In this model, the AvrPto protein is secreted by *Pseudomonas* directly into plant cells and physically interacts with the cytoplasmic kinase Pto (Scofield *et al.*, 1996; Tang *et al.*, 1996). The physical interaction of Pto and AvrPto determines the specific recognition between plants with the *Pto* gene and *Pseudomonas* bacteria carrying *avrPto*. This recognition

event, which may also involve Prf, activates the Pto kinase which in turn phosphorylates and activates diverse downstream target proteins, each with a unique role in the resistance responses. We have shown previously that Pti1 is involved in the HR mediated by *Pto–avrPto* recognition. The Pti4/5/6 proteins described here may represent components involved in a separate pathway governing defense gene expression. The occurrence of transcription factors directly downstream of the *R* gene product Pto was unexpected; however, it is not unprecedented. A similar situation occurs in the JAKs/STATs pathway governing mammalian cytokine-activated gene expression (reviewed by Ihle, 1996). JAKs are cytokine receptor-associated tyrosine kinases that phosphorylate STAT transcription factors upon cytokine activation. The phosphorylated STATs are then translocated into the nucleus where they activate interferon-responsive genes. Interestingly, in common with the two separate pathways that occur downstream of Pto, JAKs are also responsible for the activation of the distinct ERK/MAPK pathway by cytokines (reviewed by Briscoe *et al.*, 1996; Winston and Hunter, 1996).

Transcription factors are often regulated by phosphorylation. For example, activation of the mammalian transcription factor c-Jun by UV light and several other stress conditions requires the phosphorylation of two serine residues in the c-Jun transactivation domain by the JNK protein kinase (Hibi *et al.*, 1993). This phosphorylation is mediated by the direct interaction of c-Jun and JNK. Binding of Pti4/5/6 to the PR box does not appear to require phosphorylation, as shown by the DNA-binding activity of the *E.coli*-expressed proteins. However, phosphorylation of specific residues may affect nuclear localization, transcriptional activation or the interaction of Pti4/5/6 with other proteins (Hill and Treisman, 1995). The interaction of the Pto kinase with Pti4/5/6 and EREBP-2 suggests that phosphorylation of Pti4/5/6 and EREBPs may be required for their *in vivo* activity. It is noteworthy that a serine residue conserved in the putative activation domain and two threonine residues in the DNA-binding domain are conserved among Pti4/5/6 and the EREBPs. Whether these residues are phosphorylated by Pto and contribute to the activity of these transcription factors remains to be determined.

Materials and methods

Yeast two-hybrid interaction, cDNA library screening, DNA blot analysis, sequencing analysis

These experiments were performed as described previously (Zhou *et al.*, 1995).

Isolation of EREBP-1 and EREBP-2 from tobacco

The *EREBP-1* and *EREBP-2* cDNAs were amplified from tobacco W-38 using RT–PCR. An upstream primer 5′-AACCCGGGCAAAATGAATCAACCA-3′ and a downstream primer 5′-ACTCTCGAGCTCAAGAGTACCACA-3′ were used to amplify *EREBP-1*. The upstream primer 5′-CTGAATTCATGTATCAACCAATTTCGACC-3′ and the downstream primer 5′-ACTCTCGAGCAAAGTGGAGTAGTT-3′ were used to amplify *EREBP-2*. Total RNA was isolated from leaves that had been injected with 10^8 c.f.u./ml of *P.s.tabaci* (*avrPto*) 6 h post-inoculation. Five μg of total RNA were first transcribed *in vitro* using the downstream primers, and the cDNA was amplified subsequently by a standard PCR using both upstream and downstream primers. The PCR product of *EREBP-1* cDNA was digested with *Sma*I and *Xho*I, and ligated into the corresponding sites of pBluescript SK(–) (Stratagene), whereas the PCR product of *EREBP-2* cDNA was digested with *Eco*RI and *Xho*I, and ligated into the corresponding sites of pBluescript SK(–). The nucleotide sequences of *EREBP-1* and *EREBP-2* cDNA clones were verified by DNA sequencing analysis.

Expression of GST fusion proteins in E.coli

The *Pti1* cDNA was removed from the GST–Pti1 fusion plasmid (Zhou *et al.*, 1995) with *Eco*RI and *Xho*I and replaced with cDNA inserts of Pti4/5/6 or EREBP-2 to create GST–Pti4/5/6 or GST–EREBP-2 fusion constructs. *Pti4* cDNAs (nucleotides 13–993) and *Pti5* cDNA (nucleotides 82–782) were excised from pJG4-5 with *Eco*RI and *Xho*I before ligation into the pGEX vector. The full-length *Pti6* insert was PCR amplified using the full-length Pti6 cDNA clone in pBluescript SK (–) (Stratagene) as a template and the upstream primer 5′-GAGAATTCATGACGGAAAATTCAG-3′ and the T7 primer 5′-AATACGACTCACTATAG-3′. The PCR product was first partially digested with *Eco*RI and then digested completely with *Xho*I before being inserted into the GST expression vector. The PCR-amplified EREBP-2 cDNA (see above) was digested with *Eco*RI and *Xho*I and inserted into the same sites of the GST expression vector. The resulting constructs were introduced into *E.coli* strain PR745 (*lon*$^-$ New England Biolabs, Beverly, MA), and GST fusion proteins were expressed and purified as described (Guan and Dixon, 1991).

Gel mobility-shift assay

The wild-type *gln2* PR box 2×(CATAAGAGCCGCCACTAAAATAAGACCGATCAAATAAGAGCC GCCAT) and mutated PR box 2×(CATAAGATCCTCCACTAAAATAAGACCGATCAAATAAGATCCTCCAT) (Ohme-Takagi and Shinshi, 1995) were end labeled by ^{32}P as described (Ausubel *et al.*, 1994). Four fmol of probe were mixed with each of the purified GST fusion proteins in a buffer containing 2 μg of poly(dA–dT)·(dA–dT), 25 mM HEPES (pH7.5), 40 mM KCl, 0.1 mM EDTA, 10% glycerol and 1 mM dithiothreitol, incubated at room temperature for 15 min, and electrophoresed on a 4% polyacrylamide gel in 0.25× TBE buffer (Ohme-Takagi and Shinshi, 1995). The gel subsequently was dried and exposed to X-ray film.

Interaction of Pto with EREBP-2

EREBP-2 cDNA was digested with *Eco*RI and *Xho*I and inserted into the same sites on the prey vector pJG4-5. The resulting construct was introduced into EGY48 cells containing the various baits to test the activation of the *lacZ* and *LEU2* genes (Golemis *et al.*, 1994).

Plant inoculation and RNA blot analysis

Leaves of 7-week-old tobacco plants were injected with *P.s.tabaci* strain 11528R race 0 or the same strain carrying pPTE6 (Ronald *et al.*, 1992) at 10^6 or 10^8 c.f.u./ml, harvested at various time points following inoculation, and total RNA was extracted. Ten μg of RNA per sample were separated on 1% formaldehyde agarose gel, and duplicate RNA blots were hybridized to the following probes as described (Zhou *et al.*, 1995): *PRP1* (Payne *et al.*, 1989), *CHN50* (Fukuda *et al.*, 1991) and *Osmotin* (Singh *et al.*, 1989).

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