Functional Analyses of the *Pto* Resistance Gene Family in Tomato and the Identification of a Minor Resistance Determinant in a Susceptible Haplotype

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Pto is a member of a multigene family and encodes a serine/threonine kinase that mediates gene-for-gene resistance to strains of *Pseudomonas syringae* pv. *tomato* expressing *avrPto*. The inferred amino acid sequence of the *Pto* homologs from both resistant (*LpimPth2* to *LpimPth4*) and susceptible (*LescFen*, *LescPth2* to *LescPth5*) haplotypes suggested that most could encode functional serine/threonine kinases. In addition, the activation segments of the homologs are similar in sequence to that of *Pto*, and some have residues previously identified as required for binding of *AvrPto* by *Pto* in the yeast two-hybrid system. The *Pto* homologs were therefore characterized for transcription, for the ability of their products to interact with *AvrPto* in the yeast two-hybrid system, for their autophosphorylation activity, and for their potential to elicit cell death in the presence of and absence of a ligand, as well as their dependence on *Prf*. *LpimPth5*, *LpimPth4*, and *LescPth4* were not transcribed at levels detectable by reverse transcription-polymerase chain reaction. The interaction with *AvrPto* was unique to *Pto* in the yeast two-hybrid system. *LescPth2* autophosphorylated in vitro as a fusion protein. *LpimPth2*, *LpimPth3*, *LpimPth4*, *LescPth3*, and *LescPth4* did not autophosphorylate in vitro. Transient expression of wild-type *Fen* and wild-type *LpimPth3*, as well as *LescFen*, *LescPth3*, and *LescPth5* with perturbations in their P+1 loop caused cell death in *Nicotiana benthamiana*. *LpimPth3* and *LescPth3* with amino acid substitutions in the P+1 loop also elicited cell death in tomato; this was dependent on the presence of wild-type *Prf*. Consequently, some homologs could potentially encode functional resistance proteins. *LescPth5* induced cell death specifically in response to expression of *AvrPto* in tobacco in a *Prf*-dependent manner; this is consistent with a homolog from a 'susceptible' haplotype encoding a minor recognition determinant.

by corresponding resistance proteins of the plant (Baker et al. 1997; Flor 1956; Scofield et al. 1996; Tang et al. 1996). A defense response is elicited in these incompatible interactions, suppressing disease symptoms and pathogen growth (Dangl et al. 1996; Glazebrook 1999). A common characteristic of resistance is the hypersensitive response (HR) that involves rapid plant cell death at the point of infection (Goodman and Novacky 1994). In contrast, the lack of specific recognition permits a compatible interaction in which pathogen growth occurs and the plant succumbs to disease.

Classical and molecular studies have shown that resistance genes are often clustered in the genome (Michelmore and Meyers 1998). However, there are few data on the function of resistance gene homologs within a cluster. In addition to the gene conferring the primary resistance, a few homologs have been shown to confer partial resistance. *Xa21* is the member of a gene family that is necessary and sufficient to confer resistance to strains of *Xanthomonas oryzae* pv. *oryzae* expressing the cognate avirulence gene (Song et al. 1995, 1997). However, transgenic rice plants expressing another family member, *Xa21D*, exhibited partial resistance to *X. oryzae* pv. *oryzae* in a race-specific manner (Wang et al. 1998). Similarly, *Cf-9* is clustered with homologous genes in tomato and confers resistance to *Cladosporium fulvum* strains expressing *avr9* (Jones et al. 1994; Parniske et al. 1997). Two homologs of *Cf-9* conferred partial resistance in the adult tomato to isolates of *C. fulvum* with and without *avr9* (Parniske et al. 1997). Further analysis identified a naturally occurring chimeric gene *Hcr9DC* that also encoded recognition of *Avr9* (Van der Hoorn et al. 2001). In both rice and tomato, it was necessary to study the homologs independently of the primary resistance gene to reveal the abilities of the homologs to confer resistance.

Kinases are integral components of most signal transduction pathways. The catalytic core of kinases consists of approximately 300 residues encompassing twelve uninterrupted subdomains folded into a bilobal structure (Hanks and Hunter 1995). Twelve of the approximately 300 residues are essentially invariant among kinases. These twelve invariant residues and a commonality in structure have provided a basis for predicting functions and structural relationships of kinases. Many kinases are regulated through phosphorylation events in their activation segments at a sequence referred to as the P+1 loop (Johnson et al. 1996). Perturbing the activation segment, for example, by substituting the phosphorylated residue in the P+1 loop for a negatively charged amino acid such as aspartic acid or glu-

Resistance of plants to pathogenic organisms is often initiated by the recognition of avirulence proteins of the pathogen

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tamic acid can mimic activation of a kinase (Johnson et al. 1996; Rathjen et al. 1999).

Pto encodes a serine/threonine kinase and confers resistance to strains of *Pseudomonas syringae* pv. *tomato* carrying the avirulence gene, *avrPto* (Loh and Martin, 1995; Martin et al. 1993). *Pto* requires *Prf*, a nucleotide binding site-leucine rich repeat (NBS-LRR) encoding gene, to confer resistance (Salmeron et al. 1996). *Pto* is expressed in *Lycopersicon esculentum* cv. Rio Grande 76R, but the gene is absent in the two susceptible cultivars, 'Rio Grande 76S' and 'VFNT Cherry'. In contrast, *Prf* is present in all three cultivars. *Pto* interacts with *AvrPto* in the yeast two-hybrid system (Schofield et al. 1996; Tang et al. 1996) and functions in transgenic *Nicotiana benthamiana* to provide resistance to *P. syringae* pv. *tabaci* expressing *avrPto* (Rommens et al. 1995b). Aspartic acid substitutions at several positions in the P+1 loop of *Pto* resulted in constitutive gain-of-function mutants of *Pto* (Rathjen et al. 1999).

We had previously sequenced the *Pto* locus from 'Rio Grande 76R' and 'VFNT Cherry' and partially sequenced the locus of 'Rio Grande 76S' (accession numbers AF220602 and AF220603; D. Lavelle and R. Michelmore unpublished data). In 'Rio Grande 76R', the *Pto* locus contains *Prf* and a family of six homologous open reading frames (ORFs) that were introgressed from *L. pimpinellifolium*, including *Pto* and *Fen* (Fig. 1). Consistent with the naming convention proposed by Price and associates (1996), paralogs (homologs generated by a gene duplication event) of *Pto* in the resistant haplotype have been designated *LpimPth2* to *LpimPth5* (for *L. pimpinellifolium* *Pto* homologs). *LpimPth5* has two mutations causing frameshifts in its ORF and is, therefore, considered to be a pseudogene (D. Lavelle and R. Michelmore unpublished data). The *Pto* homologs of 'VFNT Cherry' and 'Rio Grande 76S' are similar and colinear with those of the resistant haplotype, except for deletions due to unequal crossing events (Fig. 1) (D. Lavelle and R. Michelmore unpublished data). These homologs are designated *LescFen* and *LescPth2* to *LescPth5* (for *L. esculentum* *Pto* homologs). Homologs with the same number are orthologs (homologs separated by a speciation event and occupying a syntenic position), as identified by sequence similarity and the same position in the haplotype. The homolog *LescPth5* was previously designated as the *pto* allele (Jia et al. 1997). However, neither susceptible *Pto* haplotype has an exact ortholog of *Pto*, due to an unequal crossover event resulting in a deletion of over half of the *Pto* orthologs in 'Rio Grande 76S' (D. Lavelle and R. Michelmore unpublished data). The haplotypes of 'VFNT Cherry' and 'Rio Grande 76S' are similar, except that homolog *LescPth3* is absent in 'Rio Grande 76S' due to a second deletion event.

The susceptible haplotype may encode a minor recognition determinant of *AvrPto*. *P. syringae* pv. *tomato* expressing *avrPto* causes enhanced necrosis on plants of both 'Rio Grande 76S' and *prf-3* (Chang et al. 2000). However, symptoms are delayed and less severe in plants of 'Rio Grande 76S' than *prf-3*. These are near-isogenic lines that differ only at the *Pto* lo-

Fig. 1. Diagram of the *Pto* locus from *Lycopersicon esculentum* cvs. Rio Grande 76R and VFNT Cherry. The arrows above and below the bar represent the arrangement of the genes in 'Rio Grande 76R' and 'VFNT Cherry', respectively. Their direction indicates the direction of transcription. p = *Lpim*, e = *Lesc*. The locus spans approximately 65 kb. Not drawn to scale.

cus; *prf-3* is a mutant line of 'Rio Grande 76R' that lacks a functional *Prf* gene (Salmeron et al. 1996) and 'Rio Grande 76S' has a functional *Prf* gene but not *Pto*. In addition, plants of 'Rio Grande 76S' respond to *Agrobacterium*-mediated transient expression of *avrPto* with a nonconfluent HR, whereas no macroscopic phenotype was observed in plants of *prf-3* (Chang et al. 2000). Therefore, one of the *Pto* homologs from 'Rio Grande 76S' or *Prf* might encode a minor recognition determinant of *AvrPto*.

To investigate the possibility of functional resistance activities at the *Pto* locus, the *Pto* homologs were characterized for activities similar to that of *Pto*. The homologs were analyzed for expression, interaction with *AvrPto* in the yeast two-hybrid system as well as in planta, and autophosphorylation activity. Substitutions were also introduced into the P+1 loop of each of the *Pto* homologs to create potential gain-of-function mutants that were then tested for elicitation of cell death in the absence of a ligand. Those that elicited cell death were also characterized for their dependence on *Prf* in tomato and tobacco.

RESULTS

Transcription of the *Pto* homologs in planta.

Polymerase chain reaction (PCR) was used to amplify reverse-transcribed mRNA from tomato cultivars Rio Grande 76R, Rio Grande 76S, and 'VFNT Cherry', in order to identify transcribed homologs of *Pto*. The mRNA samples were first assessed for DNA contamination by amplifying a portion of *Prf* with primers that span the intron located between the regions encoding the putative NBS and LRR motifs (Salmeron et al. 1996). Reverse transcription-polymerase chain reaction (RT-PCR) products of *Prf* from each of the mRNA samples were identical in size to the amplified product of a cDNA clone of *Prf* and approximately 100 bp smaller than the amplified product from genomic DNA of 'Rio Grande 76R' (Fig. 2). Therefore, the mRNA isolated from each cultivar of tomato was free from detectable amounts of DNA.

Primers that spanned the ORFs of the *Pto* homologs (Table 1) were used in RT-PCR of mRNA from each of the three genotypes. First-strand cDNA samples from each genotype were amplified with primer pairs for *Pto*, the *Pth3* orthologs, and the *Pth5* orthologs. Samples from 'Rio Grande 76R' and 'Rio Grande 76S' were also amplified with three primer pairs unique to the *Fen* orthologs, *Pth2* orthologs, and the *Pth4* orthologs. An amplification product was obtained for each pair of primers from all mRNA samples tested, except for those de-

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) of transcripts corresponding to the *Pto* homologs from tomato cultivars, Rio Grande 76R (R), Rio Grande 76S (S), and VFNT Cherry (V). This is a negative image of a 1.0%, 1× Tris-Acetate EDTA, ethidium bromide-stained agarose gel. Lanes 1 to 5 show products amplified with primers that span an approximately 300-bp genomic fragment of *Prf*; lanes 1 and 2 show products amplified from a genomic fragment and a corresponding cDNA clone of *Prf*, respectively; and lanes 3 to 5 show PCR products of reverse transcribed mRNA. A faint band of approximately 200 bp is visible in lane 5. Lanes 6 to 14 depict products from reverse transcribed mRNA. Products in lanes 6 to 8 were amplified with primers to *Pto*, the *Pth3* orthologs, and *LescPth5*. Products in lanes 9 and 10, 11 and 12, and 13 and 14 were amplified with primers to the *Fen* orthologs, *Pth2* orthologs, and *Pth4* orthologs, respectively. Lanes 15 and 16 show the products of the *Pth4* orthologs amplified from genomic DNA. One-kb ladder is designated with a "M" and sizes in kb are shown to the right.

signed to amplify *LpimPth4* and *LescPth4* (Fig. 2). All amplification products were cloned and from 15 to 20 clones were sequenced from each product. Products corresponding to *Pto*, *Fen*, *LpimPth2*, and *LpimPth3* were identified from mRNA of 'Rio Grande 76R'. However, products for *LpimPth5* were not detected using this particular primer pair. Transcripts of *LescFen*, *LescPth2*, and *LescPth5* were detected from mRNA of 'Rio Grande 76S'; *LescPth3* was transcribed in 'VFNT Cherry'. The lack of amplification of *LpimPth4* and *LescPth4* was not due to primer sequence or PCR conditions; *LpimPth4* and *LescPth4* were successfully amplified from genomic DNA samples using the same primer pair and PCR conditions. The same mRNA samples were used successfully as templates for the other homologs (Fig. 2). Consequently, the absence of amplification of the *Pth4* orthologs by RT-PCR was due to a lack of detectable transcription rather than a lack of amplification. Therefore, transcription of all *Pto* homologs was detected except for *LpimPth5*, *LpimPth4*, and *LescPth4*.

Yeast two-hybrid analysis of interactions with the *Pto* homologs.

Sequence similarities to subdomain VIII of *Pto* suggested that several homologs could potentially interact with AvrPto (Fig. 3). *LpimPth3*, *LescPth3*, and *LescPth5* have the highest overall levels of amino acid identity with *Pto*. They have the threonine in subdomain VIII that is suggested to be a critical AvrPto-binding determinant of *Pto* (Scofield et al. 1996; Frederick et al. 1998). *LpimPth4* and *LescPth4* have the lowest overall levels of amino acid identity with *Pto*. However, the translation products of both have the amino acids KxTLxxxD in subdomain VIII that were present in *Pto* but not present in *Fen* and that were required in chimeric proteins between *Fen* and *Pto* for recognition of AvrPto (Scofield et al. 1996; Tang et al. 1996). *LpimPth2* and *LescPth2* are the most divergent from *Pto* at this particular sequence.

We used the Gal4 yeast two-hybrid system to test whether any of the *Pto* homologs could interact with AvrPto. *Pto* homologs from 'Rio Grande 76R' were fused to both the Gal4 DNA-binding domain and the Gal4 DNA-activating domain, whereas *Pto* homologs from 'Rio Grande 76S' and 'VFNT Cherry' were only fused to the Gal4 DNA-binding domain. Regardless of the vector used, β -galactosidase was not activated in yeast cells cotransformed with AvrPto and any *Pto* homolog except *Pto* (Fig. 4A). Gal4 DNA-binding domain fusions with *LescFen* activated β -galactosidase in the absence of AvrPto, suggesting a nonspecific interaction with the Gal4 DNA-activating domain. Expression of each protein in yeast was confirmed by Western analysis (Chang 1999). Therefore, detectable specific interaction with AvrPto in the yeast two-hybrid system was unique to *Pto*.

To determine whether any of the *Pto* homologs from the resistant haplotype could recognize *Pto*-signaling components, each homolog was individually paired with *Pto* or *Pti1* in the yeast two-hybrid system. Again, no interactions were detected (Fig. 4B). Finally, no interactions were detected when *Pto* homologs of the resistant haplotype were paired with themselves or together with other *Pto* homologs.

In vitro autophosphorylation assays.

Analysis of the amino acid sequence of the *Pto* homologs suggested they may encode functional kinases (Fig. 3). Each homolog, with the exception of *LpimPth3* and *LescPth3*, has the twelve invariant residues characteristic of functional kinases (Hanks and Hunter 1995). *LpimPth3* and *LescPth3* have an alanine substituted for the first invariant glycine (position 48 in *Pto*). *Pto*, *Fen*, *LescFen*, and *LescPth5* had each been previously shown to autophosphorylate in vitro (Jia et al. 1997; Loh and Martin 1995; Rommens et al. 1995a).

The *Pto* homologs were fused to the maltose binding protein (MBP), partially purified, and subjected to in vitro autophosphorylation assays. Proteins were boiled and then separated in denaturing gel and reducing conditions that would disrupt all noncovalent associations. Coomassie-stained gels of all partially purified proteins showed that each was expressed and purified to detectable levels (Chang 1999). Only *Pto*, *Fen*, and *LescPth2* autophosphorylated in vitro (Fig. 5). Neither *LescFen* nor *LescPth5* were tested as they had previously been demonstrated to autophosphorylate (Jia et al. 1997). Proteins from the other homologs tested did not autophosphorylate. Therefore, in addition to *Pto*, *Fen*, *LescFen*, and *LescPth5*, *LescPth2* autophosphorylated in vitro. However, *LpimPth2*, *LpimPth3*, *LpimPth4*, *LescPth3*, and *LescPth4* did not autophosphorylate in vitro as MBP fusions.

Elicitation of cell death in planta by *Agrobacterium*-mediated transient expression of *Pto* homologs.

Elicitation of cell death by the *Pto* homologs was examined by *Agrobacterium*-mediated transient expression in *N. benthamiana*. The *Pto* homologs were fused to the CaMV35S promoter and the *Tobacco mosaic virus* RNA Ω leader sequence. DNA constructs were transformed into *A. tumefaciens* strain A281, and strains were individually infiltrated into leaves of *N. benthamiana* (Fig. 6). Cell death was observed approximately 5 days postinoculation (dpi) in leaves infiltrated with *A. tumefaciens* carrying either wild-type *LpimPth3* or *Fen*. Other wild-type *Pto* homologs did not elicit a visible phenotype when transiently expressed after infiltration with *A. tumefaciens*. T-DNA transfer and gene transcription was confirmed by assaying for GUS activity and by RT-PCR of the transgene in infiltrated leaves, respectively (Chang 1999). Therefore, of the wild-type

Table 1. Summary of oligonucleotide primers used in the analysis of the *Pto* gene family

Gene	Flanking primers ^a	Cognate to 204 substitution ^b	Cognate to 207 substitution ^b
<i>LescFen</i>	5'GGTCACCATGGGAAGCAAGTATTC 5'GTGAGAGGAGATATAGGCTAC	5'GGGTCTAGATCATTGAGTATC 5'CAGGGGCAATGTGCGCTATATTTC	5'GTAGCCTATATCTCCTCTCAC 5'GGAATATAGGCGACATTGCCCTCG
<i>LpimPth2</i> and <i>LescPth2</i>	5'CACGGATCCCATGGGAACCAACTATTCC 5'GGGTCTAGAGTCAATCAGGATTATCTG	5'GTAGCCTATATCTGGTTGCACC 5'GGTGCAAGGAGATATAGGCTAC	5'GGTCAATGTGCGCTATACTTG 5'GAAGTATAGGCGACATTGACC
<i>LpimPth3</i> and <i>LescPth5</i>	5'GGTCACCATGGGAAGCAAGTATTC 5'GGCTCTAGATTAAATAACAGACTCTTGAG	5'GTAGCCGAAATCTCCTTTACGTC 5'GACGTGAAAGGAGATTTGCGCTAC	5'CAGGGTCAATGTGCGCGAAAGTTC 5'GAACCTTTCGGCGACATTGACCTCG
<i>LescPth3</i>	5'GGTCACCATGGGAAGCAAGTATTC 5'GGCTCTAGATTAAATAACAGACTCTTGAG	5'GTAGCCGAGATCTCCTTGCACTAC 5'GTAGTGCAAGGAGATCTGCGCTAC	5'CAGGGTCAAGGTGCGCGAGAGTTC 5'GAACCTTTCGGCGACCTTGACCTCG
<i>LpimPth4</i> and <i>LescPth4</i>	5'AGAAGCAAGGATTCCAAGGAAAC 5'GCCTCTAGACTACAGCACATCACCCACAG	5'GTACCCCAATCTCCGTTTCACG 5'CGTGAAAGGAGATTGGGGTAC	5'GGTCAAGGTCCCCCAAGTT C 5'GAACCTTGGGGGACCTTGACC

^a Sequences of the primers that anneal immediately outside the ORFs.

^b Sequences of the primers used to introduce substitutions at positions cognate to 204 and 207 of *Pto* for each gene.

	subdomain I											
	102030405060											
pPth2	MG	TN	YS	SK	PT	TS	IN	DA	SN	LS	NR	VP
ePth2	MG	TN	YS	SK	PT	TS	IN	DA	SN	LS	NR	VP
eFen	MG	SK	YS	KA	TN	SI	DA	SN	LS	SY	GP	PF
Fen	MG	SK	YS	KA	TN	SI	DA	SN	LS	SY	GP	PF
pPth3	MG	SK	YS	KA	TN	SI	DA	SN	LS	SY	GP	PF
ePth3	MG	SK	YS	KA	TN	SI	DA	SN	LS	SY	GP	PF
ePth5	MG	SK	YS	KA	TN	SI	DA	SN	LS	SY	GP	PF
Pto	MG	SK	YS	KA	TN	SI	DA	SN	LS	SY	GP	PF
pPth4	MR	SK	DS	KE	TT	YI	SD	TT	SY	R	FP	VE
ePth4	MR	SK	DS	KE	TT	YI	SD	TT	SY	R	FP	VE
	subdomain II				subdomain III				subdomain IV			
	708090100110120											
pPth2	RD	GT	KV	AL	KR	HK	PE	SP	QG	IE	EF	RT
ePth2	RD	GT	KV	AL	KR	HK	PE	SP	QG	IE	EF	RT
eFen	RD	GT	KV	AL	KR	HK	PE	SP	QG	IE	EF	RT
Fen	RD	GT	KV	AL	KR	HK	PE	SP	QG	IE	EF	RT
pPth3	RD	GT	KV	AL	KR	HK	PE	SP	QG	IE	EF	RT
ePth3	RD	GT	KV	AL	KR	HK	PE	SP	QG	IE	EF	RT
ePth5	RD	GT	KV	AL	KR	HK	PE	SP	QG	IE	EF	RT
Pto	RD	GT	KV	AL	KR	NR	DS	R	SG	IE	EF	GT
pPth4	CD	GT	KV	AL	KR	CK	LE	SS	QG	IE	EF	QT
ePth4	CD	GT	KV	AL	KR	CK	LE	SS	QG	IE	EF	QT
	domain V				subdomain VIa				subdomain VIb			
	130140150160170180											
pPth2	NL	SK	RL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
ePth2	NL	SK	RL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
eFen	NL	SK	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
Fen	NL	SK	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
pPth3	NL	SK	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
ePth3	NL	SK	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
ePth5	NL	SK	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
Pto	NL	SK	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
pPth4	SL	SS	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
ePth4	SL	SS	HL	YG	SD	LC	LP	TM	SW	EQ	RL	EI
	subdomain VII				subdomain VIII				subdomain IX			
	190200210220230240											
pPth2	TD	FG	IS	KT	TP	EF	DQ	TH	LV	VQ	GS	IG
ePth2	TD	FG	IS	KT	TP	EF	DQ	TH	LV	VQ	GS	IG
eFen	TD	FG	IS	KT	TP	EF	DQ	TH	LV	VQ	GS	IG
Fen	TD	FG	IS	KT	MP	EL	DQ	TH	LV	VQ	GS	IG
pPth3	TD	FG	IS	KT	TR	PQ	LY	Q	TH	V	ST	DV
ePth3	TD	FG	IS	KT	TR	PQ	LY	Q	TH	V	ST	DV
ePth5	TD	FG	IS	KT	TR	PQ	LY	Q	TH	V	ST	DV
Pto	TD	FG	IS	KT	TR	PQ	LY	Q	TH	V	ST	DV
pPth4	TD	FE	V	SK	TR	TE	L	DQ	TH	V	ST	DV
ePth4	TD	FE	V	SK	TR	TE	L	DQ	TH	V	ST	DV
					* + *				subdomain X			
	250260270280290300											
pPth2	IG	R	...	SH	MI	SL	AE	WA	VE	TQ	KM	GL
ePth2	IG	R	...	SH	MI	SL	AE	WA	VE	TQ	KM	GL
eFen	LY	L	...	SE	MM	SS	DD	...	ET	QK	MG	LE
Fen	LDR	...	SE	IM	SL	DD	...	ET	QK	MG	LE	QI
pPth3	IV	QS	LP	SE	MV	NL	AE	WA	VE	SH	NN	GL
ePth3	IV	QS	LP	SE	MV	NL	AE	WA	VE	SH	NN	GL
ePth5	MV	QS	LP	SE	MV	NL	AE	WA	VE	SH	NN	GL
Pto	IV	QS	LP	SE	MV	NL	AE	WA	VE	SH	NN	GL
pPth4	IV	HY	IS	KG	LV	TL	AA	WA	MD	SH	KK	GL
ePth4	IG	HY	IS	KG	MV	TL	AA	WA	MD	SH	KK	GL
	domain XI											
	310320											
pPth2	PS	MG	DV	LW	KL	EY	AL	RL	QE	PI	IQ	DN
ePth2	PS	MG	DV	LW	KL	EY	AL	RL	QE	PI	IQ	DN
eFen	PS	MG	DV	LW	KL	EY	AL	CL	QE	PT	IQ	DD
Fen	PS	MG	DV	LW	KL	EY	AL	CL	QE	PT	IQ	DD
pPth3	PS	MG	DV	LW	KL	EY	AL	RL	QE	SV	...	
ePth3	PS	MG	DV	LW	KL	EY	AL	RL	QE	SV	...	
ePth5	PS	MG	DV	LW	KL	EY	AL	RL	QE	SV	...	
Pto	PS	MG	DV	LW	KL	EY	AL	RL	QE	SV	...	
pPth4	PS	VG	DV	L	...	...	...	...	...	...	...	
ePth4	PS	VG	DV	L	...	...	...	...	...	...	...	

Fig. 3. Amino acid alignment of the Pto homologs. Amino acids were aligned using ClustalX (Thompson 1997) and Macboxshade 2.15E (M. D. Baron). Gaps are represented by periods. The 12 invariant residues of a kinase are depicted by black columns. The putative activation segments are underlined with a black bar. The putative P+1 loop of each homolog begins at the position corresponding to G²⁰³ in Pto, and extends to the end of the activation segment. Variable residues between Pto and Fen found in the region previously shown to be required to confer AvrPto binding by chimeric proteins are depicted with an asterisk and positions 204 and 207 of Pto are marked with a "+" under the black bar. Putative subdomains of the Pto homologs are based on inferences from other kinases. p = Lpim, e = Lesc.

genes, overexpression of only *LpimPth3* and *Fen* elicited cell death in *N. benthamiana*.

To determine if Pto homologs could be modified to elicit cell death, amino acid substitutions were made in each of the Pto homologs that were analogous to substitutions that created ligand-independent, constitutive gain-of-function mutants of Pto (Rathjen et al. 1999). The amino acid sequences in the P+1 loop of the Pto homologs show conservation of threonines and identity of tyrosines at positions corresponding to 204 and 207, respectively, of Pto (Fig. 3). Aspartic acid substitutions at these positions in Pto resulted in mutants that constitutively elicited cell death following *Agrobacterium*-mediated transient expression assays in *N. benthamiana* (Rathjen et al. 1999).

Infiltration of *A. tumefaciens* carrying *LpimPth3*, *LescFen*, *LescPth3*, and *LescPth5* with substitutions corresponding to T204D or Y207D of Pto elicited cell death in leaves of *N. benthamiana* (Fig. 6). The leaves collapsed in a manner similar to leaves infiltrated with *A. tumefaciens* carrying either wild-type *LpimPth3* or *Pto_{Y207D}* (Rathjen et al. 1999), except the cell death responses caused by *LescPth3* and *LescPth5* with substitutions cognate to position 204 in Pto were weaker and delayed compared to that with *Pto_{Y207D}*. No cell death was observed following infiltrations of *LpimPth2*, *LescPth2*, *LpimPth4*, or *LescPth4* with substitutions in either position; transcription in these cases was confirmed by RT-PCR using primers specific to the transgene and mRNA isolated from leaves infiltrated with *A. tumefaciens* (Chang 1999).

Elicitation of cell death by *Fen*, *Pto*+*AvrPto*, and constitutive gain-of-function mutants of *Pto* are dependent on *Prf*. Therefore, we assayed the *Pto* homologs that do elicit cell death for their dependence on *Prf* in both *N. benthamiana* and tomato. Plants of *N. benthamiana* were inoculated with *Potato virus X* (PVX) expressing a 3' fragment of *Prf* derived from *N. benthamiana* to induce silencing (Baulcombe 1999) of the *Prf* gene. Three weeks later, these plants were infiltrated with *A. tumefaciens* carrying either the test constructs or *Pto_{L205D}* (as the control). None of the constructs elicited HR, demonstrating that the silencing of *Prf* was effective and that in all cases the HR elicited in wild-type plants was dependent of *Prf* (Fig. 7). In addition, these *Pto* homologs were introduced into *A. tumefaciens* strain C58C1 and infiltrated into tomato genotypes, 'Rio Grande 76R' and *prf-3*. *LpimPth3* and *LescPth3*, carrying an aspartic acid substitution at position 207, caused cell death in 'Rio Grande 76R', but no cell death was observed in *prf-3*. All other *Pto* homologs and those carrying perturbations in the P+1 loop that caused cell death in *N. benthamiana* did not exhibit phenotypes in tomato. (This was not surprising as transient assays in tomato are less reliable than those in tobacco and require lower bacterial densities than those in *N. benthamiana* to avoid a nonspecific HR induced by *Agrobacterium* alone.) These data indicate that the gain-of-function variants of the *Pto* homologs induce HR through one or more of the same signaling pathways as wild-type *Pto*.

Recognition of *AvrPto* by a *Pto* homolog in transgenic *N. benthamiana*.

In order to determine whether any of the *Pto* homologs could recognize *AvrPto* in planta, we tested them using *N. benthamiana* carrying a dexamethasone (DEX)-inducible *avrPto* transgene. Twenty-five constructs encoding each of the *Pto* homologs or their potentially gain-of-function variants were expressed in the transgenic *N. benthamiana* using *Agrobacterium*-mediated transient assays. Plants were treated with 30 μ M DEX 36 h postinoculation to induce expression of *AvrPto*. No cell death was observed with 13 of the 14 constructs that had previously failed to elicit cell death in the absence of *AvrPto*; therefore, there was no evidence that *LpimPth2*,

LpimPth4, *LescFen*, *LescPth2*, *LescPth3*, and *LescPth4* could recognize *AvrPto*. However, *LescPth5* elicited a clear HR specifically in response to the induced expression of *AvrPto* within 24 h of DEX treatment, and therefore, had a similar phenotype to *Pto* infiltrated into these plants (Fig. 8). Therefore, *LescPth5* that is present in the supposedly susceptible haplotype of tomato is capable of recognizing *AvrPto* in tobacco. To test whether this cell death was *Prf* dependent, transgenics with DEX-inducible *AvrPto* expression were inoculated with PVX expressing a 3' fragment of *Prf* from tobacco to silence the *Prf* gene. No cell death was observed in these plants following infiltration 3 weeks later with *Agrobacterium* carrying *LescPth5* or *Pto_{L205D}*, indicating that the HR induced by *LescPth5* in response to the expression of *AvrPto* is *Prf* dependent (Fig. 8).

DISCUSSION

We characterized the *Pto* homologs from both resistant and susceptible haplotypes of tomato to determine whether they have activities similar to that of *Pto* (Table 2). Based on sequence data, several *Pto* homologs could potentially encode functional resistance proteins. However, a specific recognition activity was only demonstrated for one homolog. Several lines of evidence had indicated that 'Rio Grande 76S', which is isogenic with the resistant 'Rio Grande 76R', has a minor recognition of the *AvrPto* component at the *Pto* locus but that this is not sufficient to suppress disease completely. Although 'Rio Grande 76S' is susceptible to *Pst* expressing *avrPto*, it shows less extensive symptoms relative to those observed in the mutant *prf-3* that lacks *Prf* activity (Chang et al. 2000). Similar differences were observed when 'Rio Grande 76S' and *prf3* plants were challenged with PVX::*avrPto* (Tobias et al. 1999). 'Rio Grande 76S' also shows a weak and nonconfluent HR in response to *Agrobacterium*-mediated transient expression of *AvrPto* (Chang et al. 2000).

Our current data is consistent with *LescPth5* encoding the minor recognition determinant in cv. Rio Grande 76S. When *LescPth5* was transiently expressed in *N. benthamiana*, it elicited a strong HR specifically in response to the expression of *AvrPto*. This HR was comparable in timing and intensity to the HR caused by the interaction of *Pto* with *AvrPto*. Furthermore, *LescPth5* autophosphorylated in vitro and with amino acid substitutions in the P+1 loop elicited ligand-independent cell death in *N. benthamiana*. The cell death elicited both by *LescPth5*+*AvrPto* and by the activated *LescPth5* were *Prf* dependent. Our failure to detect interactions between *LescPth5* and *AvrPto* in yeast when using the GAL 4 system and by others using the LexA system (Jia et al. 1997) may be a consequence of a lack of sensitivity of the yeast two-hybrid system to detect weak interactions. The differences in the extent of the HR observed in transient assays of transgenic *N. benthamiana* and tomato challenged with *Pst* expressing *AvrPto* may reflect differences among the experimental systems in providing different levels of both *LescPth5* and *AvrPto*. Alternatively, it may reflect differences among proteins regulating the HR response; these differences may also explain why transient expression of several *Pto* homologs resulted in cell death in *N. benthamiana* but not tomato.

LescPth5 is a chimera resulting from an unequal crossover event between progenitor sequences of *Pto* and *LpimPth5*. The crossover event occurred within the region encoding the kinase activation domain between amino acids 189 and 206, resulting in a gene that is similar to the 5' two-thirds of *LpimPth5* and the 3' third of *Pto* (Fig. 3). *LescPth5* has a 3-amino acid deletion just N-terminal to the P+1 loop compared with *Pto* and other homologs. *LescPth5* and *Pto* have the same residues at three out of four positions, equivalent to K²⁰², T²⁰⁴, and D²⁰⁹ in

Pto, that were shown to be important in recognition of AvrPto by using exchanges between Pto and Fen (Scofield et al. 1996; Tang et al. 1996; Frederick et al. 1998) and by alanine substitution analysis (Rathjen et al. 1999). LescPth5 has a phenylalanine at the position, equivalent to L²⁰⁵ in Pto, at which aspartic acid substitutions result in constitutive gain-of-function variants in Pto and several homologs, including LescPth5. These, or some of the other 49 amino acid differences between LescPth5 and Pto elsewhere in the protein, have apparently altered the ability to recognize AvrPto in the yeast two-hybrid system but have not completely abolished the recognition or the downstream signaling, as indicated by the in planta data.

The other homologs of *Pto* could also potentially encode functional resistance genes, although we have no indication of the ligand that they detect. Transcription of all genes was detected except for *LpimPth4*, *LescPth4*, and the pseudogene *LpimPth5*. Fen exhibited the majority of the tested activities of Pto; in addition, Fen elicited cell death in the absence of activating substitutions in *N. benthamiana*. LescFen exhibited similar activities to Fen except that the wild-type *LescFen* sequence did not elicit cell death in *N. benthamiana*. *LpimPth3* and *LescPth3* did not autophosphorylate but did elicit cell death as the wild-type sequence or with activating substitutions, respectively. Conversely, *LescPth2* did autophosphorylate but was not activated by the amino acid substitutions used in this study. *LpimPth2*, *LpimPth4*, and *LescPth4* had none of the tested activities, even though they had complete ORFs and had all of the 12 residues invariant in protein kinases. The *Pto* homologs that did not elicit cell death can only be considered nonfunctional to the extent that activation segment substitutions and the conditions used in this study failed to elicit cell death.

Fig. 4. Gal4 yeast two-hybrid assays of the *Pto* homologs. Yeast cells were incubated in X-Gal for no longer than 24 h. Blue cells are indicative of a positive interaction. **A**, Top row, assays for interactions between AvrPto and *Pto* homologs of the resistant haplotype; bottom row, assays for interactions between AvrPto and *Pto* homologs of susceptible haplotypes. *LpimPth5* was not tested because it is a pseudogene and LescFen interacted with an empty Gal4 DNA-activating domain (autoactivation). **B**, Assays for interactions between the *Pto* homologs of the resistance haplotype and Pti1. + = an interaction as confirmed by β -galactosidase activity; - = no detectable interaction; n.t. = could not be tested because Pto autoactivated when expressed in pACT2; p = *Lpim*; and e = *Lesc*.

The *Pto* homologs may recognize as yet unidentified ligands from *P. syringae* or from other pathogens. Transgenic tomato plants with either of two homologs of *Cf-9* conferred partial resistance to *C. fulvum* independent of *avr9* (Parniske et al. 1997). It is difficult to identify the natural ligands for putative orphan receptors such as the *Pto* homologs. It is similarly difficult, in the absence of genetic information that maps additional resistance to the *Pto* locus, to identify pathogens against which these homologs might confer resistance. Stable transgenics are of limited value in determining function of *Pto* homologs; no genetic stocks exist in which the *Pto* locus has been deleted, and therefore, disease assays would be confounded by the presence of endogenous *Pto* homologs. In addition, stable transgenics expressing *CaMV35S::LpimPth3*, *CaMV35S::Fen*, and gain-of-function mutants have reduced viability (Chang 1999).

Autophosphorylation in vitro was not always correlated with the elicitation of cell death in planta. LescPth2 autophosphorylated but did not elicit cell death. Conversely, wild-type *LpimPth3* elicited cell death even though it had no detectable in vitro autophosphorylation activity; similarly, LescPth3 did not autophosphorylate in vitro but did elicit cell death with substitutions in its P+1 loop. Therefore, the role of autophosphorylation activity in downstream signaling leading to cell death is unclear. Signaling independent of detectable autophosphorylation in vitro by a kinaselike molecule has been previously shown in the mammalian interleukin-1 receptor (IL-1R) pathway (Kopp and Medzhitov 1999). This pathway elicits innate immunity (Qureshi et al. 1999); one component, IRAK, contains a functional kinase domain with high homology to *Pto* (Cao et al. 1996). IRAK-2, a homolog of IRAK, associates with the IL-1R complex, and activates NF- κ B when ectopically expressed (Muzio et al. 1997); however, IRAK-2 lacks residues critical to kinase function and does not autophosphorylate in vitro (Wesche et al. 1999). In addition, IRAK-M, another IRAK homolog, can partially restore the IL-1 response in the absence of IRAK; it, too, does not autophosphorylate in vitro and lacks residues critical for kinase function (Wesche et al. 1999). Therefore, *LpimPth3* and *LescPth3* may belong to this class of molecules that do not require autophosphorylation activity to signal. Furthermore, our results indicate that autophosphorylation is not a prerequisite for eliciting the HR. This is contrary to the conclusions of Sessa and associates (2000). However, it is consistent with our previous observations that autophosphorylation activity was dispensable for interaction

Fig. 5. In vitro autophosphorylation assays of the *Pto* homologs. *Pto* homologs were cloned as fusions to maltose binding protein and partially purified proteins were incubated with γ^{32} P-ATP. Protein products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% acrylamide gel and assayed for autophosphorylation activity. Radiolabeled proteins were detected using a phosphorimager. Data are representative of at least three separate kinase assays; this figure is compiled from two images of kinase assays of **A**, resistant and **B**, susceptible *Pto* homologs. p = *Lpim*; e = *Lesc*.

with *avrPto* in yeast and that *Pto*_{Y207D} lacked autophosphorylation activity (Rathjen et al. 1999).

The lack of detectable autophosphorylation activity in vitro for *LpimPth2*, *LpimPth3*, and *LescPth3* might be explained by divergence from consensus sequences for kinases. Subdomain I has the motif GxGxxGxV that forms a flexible cap to hold the nontransferable phosphates of ATP (Hanks and Hunter 1995). Although not invariant, this valine is highly conserved among kinases and may contribute to a hydrophobic pocket that encloses the adenine ring of ATP (Hanks and Hunter 1995). *LpimPth2* has an aspartic acid rather than valine at this position, and this negatively charged residue may repel the negative charges of ATP. The first of the residues in the glycine triad of subdomain I is usually invariant in kinases. *LpimPth3* and *LescPth3* have an alanine instead of a glycine at this position. Alanine has a slightly larger side group than glycine, possibly adding rigidity to this motif and preventing proper orientation of ATP; substitutions from glycine to alanine at this position in mouse cAMP-dependent protein kinase decreased catalytic activity (Hemmer et al. 1997). However, we cannot exclude the possibility that the *Pto* homologs that did not show autophosphorylation activity are functional kinases but do not autophosphorylate in vitro as MBP fusion proteins.

The functional analyses of the proteins encoded by genes at the *Pto* locus support our inferences regarding the evolution of this cluster based on sequence analysis (Micheltore and Meyers 1998; D. Lavelle and R. Micheltore unpublished data). Orthologs are more similar at the sequence level than paralogs. Orthologs between resistance and susceptible haplotypes also exhibited parallel transcription and tended to have similar abilities to elicit cell death and kinase activity (Table 2). *LpimPth2* and *LescPth2* are the only orthologs that did not have similar autophosphorylation activities; however, critical kinase residues differed between these two proteins. Homologs *LpimPth4* and *LescPth4* are the most divergent members at the sequence level compared with the other *Pto* homologs. They also seem to be the least functional. Neither was transcribed at detectable levels; also, *LpimPth4* and *LescPth4* were unable to elicit cell death with substitutions in their putative activation segments and did not autophosphorylate in vitro, despite having all 12 invariant residues of serine/threonine kinases.

Recently, *Pto* homologs have been isolated from a cDNA library of *L. hirsutum* by crosshybridization to *Pto* (Riely and Martin 2001). Several of the results from the characterization of *L. hirsutum* homologs parallel ours. The orthologs *LhirPto*, *LhirFen*, *LhirPth2*, and *LhirPth5* were identified on the basis of sequence similarity to the homologs that we had characterized previously. However, orthologs *LhirPth4* and *LhirPth3* were not identified. This is consistent with little or no tran-

scription of *LhirPth4*; similarly, we did not detect transcription of *LpimPth4* or *LescPth4*. We were able to detect transcription of *LescPth3* from cv. VFNT Cherry, but not in cv. Rio Grande 76S. However, it is possible that both *LhirPth4* and *LhirPth3* are transcribed but have insufficient sequence similarity to have been isolated by crosshybridization with *Pto*. Consistent with our observations, of all the *L. hirsutum* homologs, only *LhirPto* interacted with *AvrPto* in the yeast two-hybrid system. Finally, like *LescPth2*, *LhirPth2* autophosphorylated in vitro. Together, these data indicate that not only did recognition of *AvrPto* by *Pto* arise before the diversification of *L. hirsutum* from *L. pimpinellifolium*, but that multiple members of the *Pto* gene family were present before diversification and that the spectrum of activities encoded at this locus has remained relatively static. This may indicate that there are selective advantages for the different activities of genes at this locus.

MATERIALS AND METHODS

Isolation of RNA.

Tissue (2 g) was ground in liquid nitrogen using a mortar and pestle and was vortexed in 10 ml solution D (4 M guanidinium thiocyanate, 25 mM NaCitrate, pH 7, 0.5% sodium laurylsarcosyl, 30 µl β-mercaptoethanol) that had been treated with 0.1% diethylpyrocarbonate (DEPC), 200 mM NaOAc, pH 4.5, 10 ml phenol, and 2 ml chloroform. Total RNA was precipitated in 2 M LiAc overnight at 4°C, resuspended in 0.1% DEPC-treated water, digested with 4 units of DNase I for 30 min, and treated with a 1:1 phenol/chloroform mixture. mRNA was isolated using the PolyATtract mRNA Isolation System (Promega, Madison, WI, U.S.A.). First-strand cDNA was synthesized from 100 ng of mRNA using the ProStar First-Strand kit (Stratagene, La Jolla, CA, U.S.A.). PCR was performed with gene-specific primers flanking the complete ORFs (Table 1) for 30 cycles at 92°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Products were cloned into the vector, pCR Blunt (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced.

Cloning of the *Pto* homologs and yeast two-hybrid analysis.

LescFen was amplified from cv. Rio Grande 76S using *Pfu* polymerase (Stratagene) (Table 1) and cloned into the vector, pCR Blunt (Invitrogen). *LescFen* was reamplified with primers that incorporated *NcoI* and *XhoI* restriction enzyme sites, digested with the appropriate restriction enzymes, and cloned into pAS2.1 (Clontech, Palo Alto, CA, U.S.A.) that had been digested with *NcoI* and *Sall*.

LpimPth2 was identified from a cDNA library prepared from mRNA of 'Rio Grande 76R' (J. Salmeron, unpublished data). The complete ORF was amplified with *Pfu* polymerase

Fig. 6. *Agrobacterium*-mediated transient expression of *Pto* homologs in *Nicotiana benthamiana*. The top row shows *Agrobacterium*-mediated transient expression of wild-type *Pto* homologs (WT). The second row shows *Agrobacterium*-mediated transient expression of *Pto* homologs carrying aspartic acid substitutions at positions equivalent to 204 of *Pto*, and the third row shows *Pto* homologs carrying substitutions at positions equivalent to 207 of *Pto*. HR = hypersensitive response (HR); O = lack of HR; n.t. = not tested; *p* = *Lpim*; and *e* = *Lesc*.

(Stratagene) (Table 1). An adenine was added to the blunt ends by adding 1 unit of *Taq* polymerase and incubating the reaction at 72°C for 10 min. Products were cloned into vector pCR II (Invitrogen). *LpimPth2* was then digested with *NcoI* and *EcoRI* and cloned into the corresponding sites of pAS2.1. *LescPth2* was amplified from 'Rio Grande 76S' (Table 1) using *Pfu* polymerase and cloned into pCR Blunt (Invitrogen). *LescPth2* was then digested with *NcoI* and *XhoI* and cloned into pAS2.1 that had been digested with *NcoI* and *SalI*.

The complete ORFs of *LpimPth3*, *LescPth3*, and *LescPth5* were amplified from 'Rio Grande 76R' and 'Rio Grande 76S' using *Pfu* polymerase (Table 1). *LpimPth3* and *LescPth3* were cloned into pCR2.1 (Invitrogen), after the addition of an adenine. *LpimPth3* was then digested with *NcoI* and *EcoRI* and cloned into the corresponding sites of pAS2.1. *LescPth5* was

cloned in pAS2.1 that had been digested with *SmaI* and treated with calf intestinal phosphatase. *LescPth3* was digested with *XbaI*, filled-in with Klenow according to the manufacturer's instructions (New England Biolabs, Beverly, MA, U.S.A.) and digested with *NcoI*. The insert was cloned into pAS2.1 that had been digested with *NcoI* and *SmaI*.

The complete ORFs of *LpimPth4* and *LescPth4* were amplified from 'Rio Grande 76R' and 'Rio Grande 76S', respectively, using primers 5'GGAGCTCATGAGAAGCAAGGATTCC and 5'TCTAGACTACAGCACATCACCCACAGATGG and *Pfu* polymerase. *LpimPth4* was cloned into pCR2.1 (Invitrogen) after the addition of an adenine. *LpimPth4* was then digested with *EcoRI* and ligated to pAS2.1 that had been digested with *EcoRI* and treated with calf intestinal phosphatase. The PCR product of *LescPth4* was cloned directly into pAS2.1 that had been digested with *SmaI* and treated with calf intestinal phosphatase.

Orientation of each ORF relative to the GAL4 DNA binding domain was verified by PCR and restriction digest. Both strands of all PCR products were sequenced to verify no mutations had been introduced. Yeast two-hybrid analysis was conducted using the MATCHMAKER GAL4 system following the protocols of the manufacturer (Clontech). *Pto* homologs were transformed into Y190 yeast cells. Cotransformed yeast cells were selected by growth on selective media and replicated on filters (Whatman No. 1) prior to assaying for activation of β -galactosidase using 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal).

E. coli expression and in vitro autophosphorylation assay.

The ORFs of the *Pto* homologs were cloned into pMAL-c2 (New England Biolabs) as *EcoRI-XbaI* fragments derived from each cloned PCR product.

Each was transformed into DH5 α and was expressed according to the manufacturer's instructions (New England Biolabs). Cultures were grown overnight at 37°C with shaking and were diluted 1:100 in 200 ml of Luria-Bertani (LB) media. Protein expression was induced with 1.0 mM isopropyl- β -D-thiogalactoside when cells had reached an optical density at 600 nm (OD₆₀₀) of approximately 0.4. Cells were grown for an additional 2 h at 37°C with shaking. Cells were pelleted, washed once in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride [PMSF], 5.0 μ g of leupeptin per ml, and 1.0 mM dithiothreitol [DTT]), and pelleted again. Cells were resuspended in 10 ml of column buffer and lysed by sonication. The recombinant protein was partially purified on amylose resin by batch purification.

Proteins were subjected to in vitro autophosphorylation assays (Goring and Rothstein 1992; Rathjen et al. 1999). Resin was washed twice in 200 $\times$ vol of column buffer and once with kinase buffer (30 mM Tris-HCl, pH 7.5, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.1, 10 mM MgCl₂, 2.0 mM MnCl₂ [Douville et al. 1992] with 1.0 mM PMSF and 1.0 mM DTT). The resin and retained recombinant protein was resuspended in 50 μ l of kinase buffer containing 25 μ Ci of γ ³²P-ATP (6000 Ci/mmol) and incubated at room temperature for 30 min. The resin was pelleted, washed with 200 $\times$ vol of kinase buffer, and resuspended in 20 μ l of 2 $\times$ sodium dodecyl sulfate (SDS)-loading dye. The samples were heated at 90°C for approximately 5 min and separated by SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel. The gel was dried overnight and visualized on a phosphor-imager.

Mutagenic PCR and generation of constructs for in planta transient expression.

Recombinant PCR was performed using *Pfu* polymerase and gene specific primers (Scofield et al. 1996; Table 1). Products

Fig. 7. *Prf* dependence of hypersensitive response (HR) induced by *Pto* homologs. The first column shows the reactions of wild type *Nicotiana benthamiana* (WT) without inoculation with *Potato virus X* (PVX). The second column shows the reaction of plants following inoculation with wild-type PVX. The third column shows the reaction of plants inoculated with PVX expressing a 600-bp fragment of the 3' region of *Prf* from *N. benthamiana*. HR = hypersensitive response; O = lack of HR; n.t. = not tested; *p* = *Lpim*; and *e* = *Lesc*.

were cloned into pCR Blunt (Invitrogen). Mutations were confirmed by sequencing both strands. Wild-type and recombinant sequences, except for the *Pth4* orthologs, were digested with *NcoI* and *XbaI* and ligated to compatible sites in pSLJ4D4 between the CaMV35S:: Ω and *ocs3'* sequences (Jones et al. 1992). Clones were then digested with *EcoRI* and *HindIII*, and the entire expression cassette was ligated to compatible sites in the binary T-DNA vector, pTFS40 (British Sugar Corp., Norwich, U.K.).

The PCR products of the *Pth4* orthologs were digested with *XbaI*. Vector pSLJ4D4 was digested with *NcoI*, filled-in with Klenow, and digested with *XbaI*. Digested PCR prod-

ucts of the *Pth4* orthologs were then cloned into the digested pSLJ4D4 vector. This created an in-frame fusion starting at the second codon of the *Pth4* orthologs. Mutations were confirmed by sequencing both strands. The *Ocs 3'* sequence of pSLJ4D4 was digested with *XbaI* and *HindIII* and ligated to compatible sites in pTFS40. Wild-type *LpimPth4* and mutagenized clones in pSLJ4D4 were then digested with *EcoRI* and *XbaI* and ligated upstream of the *Ocs 3'* sequence in pTFS40. Wild-type *LescPth4* and mutagenized ORFs in pSLJ4D4 were then digested with *XbaI* and partially digested with *EcoRI* and ligated upstream of the *Ocs 3'* sequence in pTFS40.

Fig. 8. Recognition of AvrPto by *LescPth5* in *Nicotiana benthamiana*. Homozygous transgenic plants of *N. benthamiaina* line 291-2 with a dexamethasone (DEX)-inducible *avrPto* transgene were inoculated with either PVX, PVX carrying a 600-bp fragment of *Prf* from *N. benthamiana*, or left uninoculated. After 3 weeks, previously uninoculated leaves were infiltrated with *Agrobacterium* carrying the empty vector pTFS40 (negative control), *Pto* (positive control for the expression and specific recognition of AvrPto), *Pto*_{L205D} (positive control for virus-induced gene silencing of *Prf*), and *LescPth5*, as well as other homologs (not shown). Plants were either mock inoculated (–) or treated with 30 μ M DEX (+) 36 h after infiltration.

Table 2. Activities of the Pto homologs

Homolog	Transcription	Interaction with		AvrPto dependent HR in planta ^c	HR induction in planta ^d		
		AvrPto in Y2H ^a	Autophosphorylation ^b		WT	Δ 204	Δ 207
Pto	+ ^e	+ ^f	+ ^g	+	– ^h	+ ^h	+ ^h
Fen	+ ^e	– ^f	+ ^g	n.t.	+	n.t.	n.t.
LpimPth2	+	–	–	–	–	–	–
LpimPth3	+	–	–	n.t.	+	+	+
LpimPth4	–	–	–	–	–	–	–
LpimPth5	–	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Lescfen	+ ⁱ	–	+ ⁱ	–	–	+	+
LescPth2	+	–	+	–	–	–	–
LescPth3	+	–	–	–	–	+	+
LescPth4	–	–	–	–	–	–	–
LescPth5	+ ⁱ	–	+ ⁱ	+	–	+	+

^a + = interacted with AvrPto; – = did not interact with AvrPto.
^b + = autophosphorylated in vitro; – = did not autophosphorylate in vitro.
^c + = elicited cell death in transgenic *Nicotiana benthamiana* after treatment with 30 μ M dexamethasone, – = no cell death,
^d + = elicited cell death in *N. benthamiana*; – = did not elicit cell death; n.t. = not tested, either a pseudogene or the wild type induced HR in the absence of AvrPto.
^e Martin et al., 1993.
^f Scofield et al. 1996; Tang et al. 1996.
^g Loh and Martin 1995.
^h Rathjen et al. 1999.
ⁱ Jia et al. 1997.

Generation of transgenic plants with DEX-inducible expression of AvrPto.

The *avrPto* gene was amplified by PCR using KlenTaq (Clontech) and Pfu (Stratagene) polymerases and oligonucleotide primers to introduce terminal XhoI and Spe sites and an N-terminal HA-tag to the translated protein (5'CTCGAGATGGGAAATATATGTGTCGGCGG and 5'AC-TAGTTCAAGCGTAATCTGGAACATCGTATGGGTATTGC-CAGTTACGGTA), cloned into pTOPO (Invitrogen), and then subcloned as an XhoI-Spe fragment into pTA7002 (Aoyama and Chua 1997). This binary vector carrying the AvrPto gene was transformed into the *Agrobacterium* strain LBA4404 by the freeze and thaw method. *N. benthamiana* plants were transformed as described by Horsch and associates (1985). Several independent transgenic lines were obtained. Southern and segregation analyses confirmed the presence of a single T-DNA insertion in line 291-2. This line was selfed to provide homozygous families for use in this study. The induction of AvrPto expression only upon application of 30 μ M DEX was confirmed by Western blots using AvrPto antibody, as well as by the lack of HR induction by Pto in the absence of DEX.

Agrobacterium-mediated transient expression in planta.

DNA constructs were transformed by the freeze and thaw method or electroporation into *A. tumefaciens* strains A281 or C58C1 for infiltration of *N. benthamiana* or tomato respectively. For transient expression assays, overnight cultures were diluted 1:10 in LB media, 200 μ M acetosyringone, and 20 μ g of kanamycin per ml, plus 5 μ g of tetracycline per ml for strain C58C1, and grown at 30°C overnight. Cultures were washed in infiltration media ($^{1/10\times}$ Murashige-Skoog [MS] salts, $^{1/10\times}$ B5 vitamins, 20 mM MES [morpholineethanesulfonic acid], 2% [wt/vol] sucrose, 1% [wt/vol] glucose, 200 μ M acetosyringone, pH 5.4) and diluted to an OD₆₀₀ of 1.0 or 0.3 for *N. benthamiana* or tomato, respectively. *A. tumefaciens* was pressure infiltrated, using a syringe, into leaves of approximately 6-week-old *N. benthamiana* plants or 4-week-old tomato plants. Plants were grown at 21°C for 4 to 5 days and then scored for the presence of cell death. For analysis of interactions with AvrPto in planta, AvrPto expression was induced in the transgenic *N. benthamiana* plants 36 h after infiltration with *Agrobacterium* by applying a 30 μ M DEX solution with a cotton swab to the upper surface of the leaves. The photographs in Figures 6, 7, and 8 were taken 5 dpi; the results shown are representative of at least three separate infiltrations.

Virus-induced gene silencing (VIGS) of Prf.

In order to generate plants of *N. benthamiana* lacking expression of *Prf*, a 600-bp fragment of *Prf* was amplified from *N. benthamiana* by PCR, using primers designed from the 3' part of the *Prf* ORF from tomato that introduced terminal *Cla*I sites (5'-CCATCGATGCCCTTCTGTGGCGCCCATC-3' and 5'-CCATCGATGCTTGAGGAAGTCAAGGTGGCT-TAG-3'). This was cloned as a 600-bp fragment into *Agrobacterium* binary vector pGR106 which contains PVX downstream of a 35S promoter (Baulcombe et al. 1995). Single leaves of 4-week-old plants were infiltrated with *Agrobacterium* strain C58C1 carrying the construct. Infected plants developed mottled symptoms characteristic of PVX infection throughout the plant. Three weeks after infection, transient assays were performed as above by infiltrating younger leaves than those originally inoculated with PVX. VIGS was confirmed in every experiment by infiltration with *Agrobacterium* strains carrying the constitutively HR-inducing Pto_{L205D}.

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