Thr38 and Ser198 are Pto autophosphorylation sites required for the AvrPto–Pto-mediated hypersensitive response

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The tomato Pto kinase confers resistance to *Pseudomonas syringae* pv. tomato expressing the AvrPto protein. To elucidate the role of Pto autophosphorylation in disease resistance, eight sites autophosphorylated by Pto *in vitro* were identified by a combination of HPLC purification of tryptic phosphopeptides, MALDI-TOF/MS analysis and Edman degradation. Mutational analysis of the autophosphorylation sites revealed that Pto residues Thr38 and Ser198 are required for AvrPto–Pto-mediated elicitation of a hypersensitive response in the plant. Thr38, which is the main Pto autophosphorylation site and is located outside the kinase catalytic domain, was also required for Pto kinase activity and its physical interaction with AvrPto, the Pti1 kinase and the transcription factor Pti4. Ser198, located in the Pto activation domain, was dispensable for kinase activity and for interaction with AvrPto. However, a mutation at this site resulted in altered Pto interactions with the Pti1 kinase and the Pto interactors of unknown function Pti3 and Pti10. These results suggest that autophosphorylation events at Pto Thr38 and Ser198 are required for signal transduction by Pto and participate in distinct molecular mechanisms.

Keywords: autophosphorylation/hypersensitive response/Pelle/IRAK/tomato/*Pseudomonas syringae*

Introduction

Plants have evolved sophisticated molecular strategies to detect pathogen infection efficiently and deploy a wide array of defense responses culminating in disease resistance (Hammond-Kosack and Jones, 1996). A major system responsible for pathogen detection consists of a set of plant proteins, encoded by resistance (*R*) genes, that specifically recognize pathogen proteins encoded by avirulence (*avr*) genes. In many instances, this specific gene-for-gene interaction triggers the elicitation of a rapid and localized cell death at the site of pathogen infection known as the hypersensitive response (HR).

In recent years, several *R* genes conferring resistance to specific bacteria, fungi, viruses or nematodes have been isolated from both dicots and monocots (Hammond-Kosack and Jones, 1997). Insights into structural and functional properties of *R* gene products confirmed their

proposed function as receptors and signal transducers (Martin, 1999). A direct role in recognizing an Avr product is demonstrated only for the Pto protein, which does not contain an obvious receptor-like domain. Pto is a serine/threonine kinase that confers resistance to *Pseudomonas syringae* pv. tomato expressing the AvrPto protein (Martin *et al.*, 1993). *Pseudomonas syringae* is the causal agent of speck disease in tomato, and during pathogenesis is thought to deliver the AvrPto protein into plant cells via a type III secretion system (Galan and Collmer, 1999). A physical interaction between the Pto kinase and AvrPto is observed in a yeast two-hybrid system, and is strictly correlated with the onset of disease resistance in plants (Schofield *et al.*, 1996; Tang *et al.*, 1996). The Pto kinase activation domain plays a critical role in AvrPto recognition. In this region, Thr204 is required for recognition specificity (Frederick *et al.*, 1998), while mutations at Tyr207 eliminate the need of AvrPto for Pto activation (Rathjen *et al.*, 1999). However, elicitation of the HR by Pto proteins that are mutated at Tyr207 still requires the Prf protein, which appears to act downstream of the AvrPto–Pto recognition in the signaling events leading to speck disease resistance (Rathjen *et al.*, 1999).

Relatively little is known about mechanisms of *R* protein activation and downstream signal transduction events (Baker *et al.*, 1997; Martin, 1999). The enzymatic activities of the Pto kinase and the receptor-like kinase (RLK) Xa21 suggest that phosphorylation may be implicated in their activation and in the signaling pathways they initiate (Martin *et al.*, 1993; Song *et al.*, 1995; Sessa and Martin, 2000). Several lines of evidence indicate that kinase signaling cascades may also originate downstream of other *R* proteins. A class of plant *R* genes, including tobacco *N*, flax *L6* and *Arabidopsis* *RPP5*, share homologous domains with the interleukin-1 receptor in mammals and Toll in *Drosophila*, which transduce extracellular signals by activating phosphorylation cascades (O'Neill and Greene, 1998). In addition, the tobacco mitogen-activated protein kinases WIPK and SIPK are activated by gene-for-gene interactions that mediate resistance to tobacco mosaic virus (TMV) and to the fungal pathogen *Cladosporium fulvum* (Zhang and Klessig, 1998; Romeis *et al.*, 1999).

Phosphorylation appears to be of central importance in the AvrPto–Pto system. Pto is a functional serine/threonine kinase (Loh *et al.*, 1995), and autophosphorylates *in vitro* at multiple sites by an intramolecular mechanism (Sessa *et al.*, 1998). Pto kinase activity is required for the elicitation of the hypersensitive response and for its physical interaction with components of the same pathway (Zhou *et al.*, 1995, 1997; Schofield *et al.*, 1996; Tang *et al.*, 1996; Rathjen *et al.*, 1999). A target of Pto phosphorylation is the serine/threonine kinase Pti1, which is phosphorylated *in vitro* by Pto in the kinase

activation domain (Zhou *et al.*, 1995; Sessa *et al.*, 2000). In addition, Pti1 phosphorylation is required for the physical interaction between the two proteins (Sessa *et al.*, 2000). Phosphorylation by Pto, and the finding that its overexpression in tobacco accelerates the elicitation of an HR, support a function for Pti1 as a downstream effector of Pto (Zhou *et al.*, 1995). An additional substrate for Pto phosphorylation is the transcription factor Pti4, which physically interacts with Pto in a yeast two-hybrid system (Zhou *et al.*, 1997; Gu *et al.*, 2000). The proposed function of Pti4, based on its DNA-binding properties, is to activate transcription of a subset of pathogenesis-related genes, following its activation by Pto phosphorylation.

Autophosphorylation has been recognized to have important regulatory functions for many protein kinases. In several instances, it determines critical structural changes in the proximity of the catalytic core of the enzyme that result in induction of its kinase activity (Johnson *et al.*, 1996). Autophosphorylation may also have an inhibitory effect on kinase activity, as observed for members of the casein kinase family (Fish Gietzen and Virshup, 1999). In addition, it has been found to modulate protein-protein interactions such as those involving the *Arabidopsis* receptor-like kinase RLK5 and the *Drosophila* Pelle kinase (Stone *et al.*, 1994; Shen and Manley, 1998).

Autophosphorylation of the Pto kinase might play a role in different steps of the AvrPto-Pto signaling pathway, including Pto recognition of AvrPto, Pto activation or interactions with downstream effectors. To study the role of Pto autophosphorylation in the molecular mechanisms that lead to speck disease resistance in tomato, we first identified sites of Pto autophosphorylation *in vitro*. We then investigated the role of these sites in the elicitation of the hypersensitive response, in Pto kinase activity and in protein-protein interactions. This analysis revealed that Pto autophosphorylation sites Thr38 and Ser198 are required for the elicitation of the hypersensitive response and participate in distinct molecular mechanisms.

Results

Analysis of a Pto tryptic digest by reverse-phase HPLC

The serine/threonine protein kinase Pto autophosphorylates *in vitro* at multiple sites (Sessa *et al.*, 1998). In an effort to identify Pto autophosphorylation sites and examine their role in Pto function, we first isolated a large quantity of phosphopeptides derived from a tryptic digest of autophosphorylated Pto. A glutathione S-transferase (GST)-Pto fusion protein was expressed in bacteria, autophosphorylated *in vitro* in the presence of [γ - 32 P]ATP and digested by trypsin. A tryptic digest of autophosphorylated GST-Pto was then separated by reverse-phase HPLC, as shown in Figure 1. Fractions were collected at 30 s intervals and their radioactivity determined by Cerenkov counting. Eleven peaks with a radioactivity content above background were detected (A-K in Figure 1). Among them, G was the major peak, with an HPLC retention time of 60 min, and contained ~20% of total radioactivity. Peaks B, E, H and I, with retention times of 38, 53.5, 64 and 67.5 min, respectively,

Fig. 1. Reverse-phase HPLC fractionation of a tryptic digest of autophosphorylated GST-Pto. A tryptic digest of autophosphorylated GST-Pto was fractionated by reverse-phase HPLC using a C18 column. Peptides were eluted by a gradient of buffer B, containing 0.01% TFA, 75% acetonitrile and 25% water (dotted line). The radioactivity of each fraction was determined by Cerenkov counting (circles).

had an intermediate radioactivity content between 5 and 10%, while peaks A, C, D, F, J and K (retention times of 37, 45.5, 51, 58, 73 and 77 min, respectively) contained a low level of radioactivity with <3% of total c.p.m. The number of detected peaks and their degree of radioactivity were consistent with the pattern of phosphopeptides observed in two-dimensional phosphopeptide maps of tryptic digests of MBP-Pto (Sessa *et al.*, 1998) and GST-Pto (data not shown). Each one of the peaks could be derived either from one or more Pto tryptic phosphopeptides, with single or multiple phosphorylation sites.

Identification of Pto autophosphorylation sites

In order to determine the identity of Pto tryptic phosphopeptides, all the radioactive peaks in Figure 1, except for C, J and K, were purified further by reverse-phase HPLC, by using a slow acetonitrile gradient and pooling radioactive fractions (data not shown). For peak C, the low level of radioactivity hindered further purification and characterization. Peaks J and K were not considered for further analysis, as their radioactivity was only 2-fold higher than background, and they were not observed consistently in different HPLC runs. In addition, their late retention time and the high concentration of acetonitrile required for their elution suggested that they contained partially digested phosphopeptides. After a second HPLC purification, each one of the other peaks was analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF/MS). The observed masses in the MALDI-TOF/MS spectra were compared with predicted masses of possible GST-Pto tryptic peptides below m/z 3000 (including unphosphorylated, phosphorylated, oxidized and partially digested forms). As a representative example of MALDI-TOF/MS analysis, the spectrum of peak G is shown in Figure 2A. Two major components of m/z 1833.7 and 1922.4, differing in mass by 89 atomic mass units (amu), were detected in this spectrum. A difference of 89 amu in our analysis represented the loss of a phosphate group during the run, as observed for a synthetic phosphoserine peptide used as a standard (data not shown). The 1922.4 mass corresponded to the phosphorylated form of the tryptic peptide 29-44 (Table I), and the 89 mass difference suggested that the lighter peptide was derived by dephosphorylation of the

Fig. 2. Identification of Pto autophosphorylation sites. **(A)** MALDI-TOF/MS spectrum of HPLC-purified peak G, derived from a tryptic digest of autophosphorylated GST-Pto. *m/z* values for the major components of the spectrum, and the sequence of the phosphopeptide identified are indicated. **(B)** Reverse-phase HPLC fractionation of a tryptic digest of autophosphorylated GST-Pto(T288A). A tryptic digest of autophosphorylated GST-Pto(T288A) was fractionated by reverse-phase HPLC using a C18 column. Peptides were eluted by a gradient of buffer B, containing 0.01% TFA, 75% acetonitrile and 25% water (dotted line). The radioactivity of each fraction was determined by Cerenkov counting (circles). **(C)** Manual Edman degradation of phosphopeptide 8–28. The partially purified phosphopeptide 8–28, detected in peak I, was immobilized on a membrane disk and subjected to sequential cycles of Edman degradation. Bars represent the amount of radioactivity released after each cycle. The amino acid sequence of peptide 8–28 is shown on top of the diagram.

heavier one. Sequencing of the purified peptide from peak G by automated Edman degradation also confirmed the finding of the MALDI-TOF/MS analysis, and phosphoamino acid analysis revealed that a phosphothreonine is the source of the radioactivity of this phosphopeptide. Pto Thr38 was thus identified as the phosphorylated

residue, since it is the only threonine present in this peptide. The results of the analysis of radioactive peaks, derived from the HPLC fractionation shown in Figure 1, are described below and summarized in Table I and Figure 3.

Peaks A and B. MALDI-TOF/MS analysis of peaks A and B, for unknown reasons, did not identify any phosphopeptide. However, by using different techniques, we were able to determine the identity of the peptides present in these fractions. By phosphoamino acid analysis, it was found that a threonine is the phosphorylated residue in both peptides, which appeared to be small and hydrophilic, because of their early elution from the HPLC column. In addition, their very similar retention time suggested that they could be partial digests of the same peptide. The Pto tryptic peptide 285–291 was found to match these characteristics, being small, hydrophilic and containing a threonine residue. Moreover, it is preceded by consecutive arginine and lysine residues that could cause its partial digestion to the two very similar peptides 285–291 and 284–291 (Table I). To test this hypothesis, Thr288 was mutated to alanine by site-directed mutagenesis, and a tryptic digest of autophosphorylated Pto(T288A) was analyzed by HPLC. As shown in Figure 2B, radioactive peaks A and B were not present in the HPLC fractionation of a Pto(T288A) tryptic digest, strongly suggesting that Thr288 is the phosphorylated residue in the phosphopeptides present in these fractions.

Peaks D and E. The MALDI-TOF/MS spectrum of peak D exhibited masses corresponding to peptide 189–202 and to its partial digest 188–202, in their unphosphorylated and phosphorylated forms, with one, two or three phosphate groups (Table I). Mass spectrometry analysis of peak E identified masses corresponding to peptide 189–202, also present in peak D, with the addition of one, two or four phosphate groups. Taken together, these results suggest that four sites are autophosphorylated in peptide 189–202, and these are Thr190, Thr195, Ser198 and Thr199 in the Pto sequence (Figure 3).

Peaks F and G. MALDI-TOF/MS analysis of peak F revealed the presence in this fraction of phosphopeptide 29–44 phosphorylated at Thr38, which is also the phosphopeptide of peak G, as described above. Although it is unclear why a small portion of this phosphopeptide is released earlier than the bulk of it, this could be related to weak interactions with the column or with additional peptides in the digest.

Peak H. Analysis of peak H by mass spectrometry detected multiple masses corresponding to tryptic peptide 125–141, and representing different forms of the same peptide that derived from phosphorylation at one site, dephosphorylation and multiple oxidation events. Thr133 was identified as the phosphorylation site in peptide 125–141, since only phosphothreonine was detected by phosphoamino acid analysis of this peptide that contains only one threonine (Table I).

Peak I. The mass of peptide 8–28, phosphorylated at one site, was identified in peak I by MALDI-TOF/MS analysis. By phosphoamino acid analysis, a serine was determined as the phosphorylated residue in this peptide. In order to

Table I. Phosphopeptides identified in a tryptic digest of *in vitro* autophosphorylated GST-Pto

Peak ^a	P-AA ^b	Determined by	Sequence	No. of P-groups	Site(s) identified
A and B	P-Thr	Phosphopeptide map of Pto(T288A) mutant	285FGDTAVK291	1	Thr288
C			284KFGDTAVK291		ND
D		MALDI-TOF/MS	189GTELDQTHLSTVVK202	1, 2 or 3	ND
			188KGTELDQTHLSTVVK202	1, 2 or 3	
E		MALDI-TOF/MS	189GTELDQTHLSTVVK202	1, 2 or 4	Thr190, Thr195, Ser198, Thr199
F	P-Thr	MALDI-TOF/MS	29VPLVDLEEATNNFDHK44	1	Thr38
G	P-Thr	MALDI-TOF/MS, automated Edman degradation	29VPLVDLEEATNNFDHK44	1	Thr38
H	P-Thr	MALDI-TOF/MS	125HLYGSDLPTMSMSWEQR141	1	Thr133
I	P-Ser	MALDI-TOF/MS, manual Edman degradation	8ATNSINDALSSSYLVFPESYR28	1	Ser17
J					ND
K					ND

^aRadioactive peaks detected in HPLC fractionation of a tryptic digest of autophosphorylated Pto, as indicated in Figure 1.

^bPhosphoamino acids determined as described in Materials and methods.
ND, not determined.

test which of the five serines of peptide 8–28 was phosphorylated, the purified phosphopeptide was immobilized on a solid support and subjected to sequential cycles of manual Edman degradation. A major release of radioactivity was observed at cycle 10, identifying Ser17 as the phosphorylated residue of tryptic peptide 8–28 (Figure 2C; Table I).

Taken together, the analysis of radioactive fractions detected by HPLC analysis of a tryptic digest of autophosphorylated GST-Pto identified eight autophosphorylation sites in the Pto amino acid sequence (Figure 3; Table I). Two sites, Ser17 and Thr38, which *in vitro* showed the highest incorporation of radioactivity, are located at the N-terminus of Pto, outside the kinase catalytic domain. Six additional autophosphorylation sites, Thr133, Thr190, Thr195, Ser198, Thr199 and Thr288, were identified within the Pto catalytic domain. Remarkably, among them, Thr190, Thr195, Ser198 and Thr199 are located in the kinase activation domain, which is defined in all protein kinases as the region between the conserved motif DFG and PE of kinase subdomain VII and VIII (Hanks and Quinn, 1991; Johnson *et al.*, 1996).

In order to examine the conservation of residues identified as Pto autophosphorylation sites in homologous protein kinases, we compared Pto with the product of a non-functional *Pto* allele, the tomato Fen, the rice Xa21, the human IRAK and the *Drosophila* Pelle (Table II). The most relevant findings of this comparison are the perfect conservation in all these proteins of a threonine residue, which aligns with the main Pto autophosphorylation site Thr38, and the complete lack of conservation observed for Ser17, Thr133 and Thr190.

Pto autophosphorylation sites Thr38 and Ser198 are required for the elicitation of the AvrPto–Pto-mediated hypersensitive response

Transient expression in *Nicotiana benthamiana* plants of Pto from tomato and AvrPto from *P.syringae* triggers a hypersensitive response (HR; Scofield *et al.*, 1996; Tang *et al.*, 1996). To examine *in vivo* their role in the ability of Pto to elicit an HR, the Pto autophosphorylation sites were mutated individually to alanine by site-directed muta-

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1  MGSKYSKATNSINDALSSSYLVFPESYRNVPLVDLEEATNNFDHKFLIGHG
51  VFQKVYKGVLRDGAVALKRRTPESSQGIEEFETEIETLSFCRHPHLVSL
   binding site
101 IGFCDERNEMILYKYMENGNLKFLHYGSDLPTMSMSWEQRLEICIGAAR
151 GLHYLHTRAIHRDVKSINILLDENFVPKITDFGISKKGTTELDQTHLSTV
   Activation domain
201 VKSTLGYIDPEYFIKGRLTEKSDVYSFGVVLFEVLCARSAIVQSLPREMV
251 NLAEWAVESHNNQLEQIVDPNLADKIRPESLRKFGDTAVKCLALSSDDR
301 PSMGDLVWKLEYALRLQESVI

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Fig. 3. Phosphopeptides and autophosphorylation sites identified in the Pto amino acid sequence by analysis of a GST-Pto tryptic digest. Sequences of tryptic phosphopeptides are boxed, and residues identified as autophosphorylation sites are in bold. Glycine residues of the conserved motif GXGXXG for the ATP-binding site, and the DFG and PE motifs representing the borders of the kinase activation domain are underlined (Hanks and Quinn, 1991).

genesis. Pto autophosphorylation mutants were then introduced into the binary vector pBTEX under control of the cauliflower mosaic virus (CaMV) 35S promoter. A construct for the expression of AvrPto driven by a separate CaMV 35S promoter was also present in the same vector. The plasmids obtained were transformed into *Agrobacterium tumefaciens* strain EHA105, which was then infiltrated into mature leaves of *N.benthamiana*. In a typical experiment, 6–8 leaves of three different plants were infiltrated. Each leaf contained a negative control expressing only AvrPto, two positive controls expressing wild-type Pto and AvrPto, and three replicates expressing the autophosphorylation mutant to be tested and AvrPto (Figure 4A and B). An HR was normally observed in the positive control 3–5 days after infiltration, and an HR index was calculated for each mutant, based on the severity of the symptoms it developed, as compared with wild-type Pto. As shown in Figure 4A–C, a mutation of Pto Thr38 or Ser198 to alanine completely abolished the ability of Pto to induce an HR. Introduction of the same substitution at Thr199 or Thr288 resulted in a partial reduction in the elicitation of an HR (74 and 44% compared with wild-type Pto). No significant effect was observed by introducing mutations at autophosphorylation sites Ser17, Thr133, Thr190 and Thr195 (Figure 4C).

Table II. Conservation of residues identified as Pto autophosphorylation sites in Pto-homologous kinases from different organisms

Protein ^a	Ser17	Thr38	Thr133	Thr190	Thr195	Ser198	Thr199	Thr288
pto	— ^b	+	—	—	+	—	+	+
Fen	—	+	—	—	+	+	+	+
Xa21	—	+	—	—	—	+	+	—
IRAK	—	+	—	—	—	—	—	—
Pelle	—	+	—	—	—	—	—	—

^aThe Pto-homologous protein kinases examined are the product of a *Pto* allele non-functional in disease resistance (pto; DDBJ/EMBL/GenBank accession No. U59316), the kinase Fen (U59317), the rice receptor-like kinase Xa21 (U37133), the IRAK kinase from mammals (L76191), and the kinase Pelle from *Drosophila* (L08476).

^bThe '—' and '+' signs represent, respectively, the absence and presence of an identical residue at the corresponding position in Pto.

Fig. 4. Elicitation of the hypersensitive response (HR) by expression of Pto autophosphorylation mutants and AvrPto in *N.benthamiana* leaves. (A) Transient expression assay of Pto(T38A) for the HR in *N.benthamiana* leaves. Mature leaves were infiltrated with *A.tumefaciens* EHA105 strains (OD₆₀₀ = 0.4) that contained the pBTEx plasmid expressing the following combinations of proteins: in 1, 2 and 3, Pto(T38A) and AvrPto; in 4, AvrPto; and in 5 and 6, Pto and AvrPto. The HR was visualized as localized tissue collapse typically within 3–5 days. (B) Transient expression assay of Pto(S198A) for the HR in *N.benthamiana* leaves. Mature leaves were infiltrated as described in (A), with *A.tumefaciens* strains expressing the following combinations of proteins: in 1, 2 and 3, Pto(S198A) and AvrPto; in 4, AvrPto; and in 5 and 6, Pto and AvrPto. (C) An HR index, reflecting the efficiency of the indicated mutants in the elicitation of the HR *in vivo*, was calculated as described in Materials and methods. Calculation of the HR index included at least 82 independent observations for each mutant.

Taken together, these results indicate that Pto Thr38 and Ser198, which are autophosphorylation sites *in vitro*, are required for the elicitation of the HR in plants. In addition, autophosphorylation sites Thr199 and Thr288, although not required, participate in the induction of the HR.

Tyr207 in the Pto activation domain has been shown previously to be a negative regulator of Pto activity (Rathjen *et al.*, 1999). In fact, the substitution of this residue by aspartate, tryptophan or alanine caused the induction of an HR in the absence of AvrPto. Since four Pto autophosphorylation sites are similarly located in the kinase activation domain, the Pto autophosphorylation mutants were tested for their ability to induce an HR in the absence of AvrPto. None of the Pto mutant forms showed a constitutive gain-of-function phenotype (data not shown), indicating that the Pto autophosphorylation sites do not exert a negative effect on Pto function.

In several instances, the introduction of a negatively charged residue at certain sites has been shown to be a functional mimic of phosphorylation (Johnson *et al.*, 1996). To investigate further the role of autophosphorylation at Thr38 and Ser198, we tested the effect of introducing a negatively charged residue at these sites on the elicitation of the HR. Thr38 and Ser198 were substituted individually to aspartate, and the Pto mutants obtained were expressed in *N.benthamiana*, with or without AvrPto. Both Pto(T38D) and Pto(S198D) were unable to induce an HR, in the absence as well as in the presence of AvrPto (data not shown). These results suggest that a negative charge at Thr38 and Ser198 is not sufficient to mimic the function of these autophosphorylation sites in the elicitation of the HR.

A mutation at Thr38, the main Pto autophosphorylation site, abolishes Pto kinase activity

Pto kinase activity has previously been shown to be required for the elicitation of the HR in *N.benthamiana* and tomato plants (Scofield *et al.*, 1996; Tang *et al.*, 1996; Rathjen *et al.*, 1999). This raised the possibility that the inability of Pto(T38A) and Pto(S198A) to elicit an HR was related to a defect in their kinase activity. We thus tested whether mutations at specific autophosphorylation sites affected Pto kinase activity. To this end, Pto forms individually mutated at autophosphorylation sites were expressed in *Escherichia coli* as GST fusion proteins and purified. The mutant proteins were tested *in vitro* in a qualitative assay for their autophosphorylation activity, and for the ability to phosphorylate the serine/threonine kinase Pti1 (Zhou *et al.*, 1995), or the transcription factor Pti4 (Zhou *et al.*, 1997). As observed by Coomassie staining after electrophoresis (Figure 5A and B, bottom panels), the mutant Pto forms were expressed in bacteria at similar levels. However, Pto(T38A) consistently migrated in SDS-PAGE gels with a slightly faster mobility, as compared with the other mutants and wild-type Pto, possibly related to its phosphorylation state. Consistent with this observation, Pto(T38A) was the only Pto form that completely lost its ability to autophosphorylate, as the result of a specific mutation of an autophosphorylation site (Figure 5A). In addition, the substitution of Thr38 with an alanine also abolished the ability of Pto to phosphorylate substrate proteins. Pto(T38A) failed to phosphorylate a

Fig. 5. *In vitro* kinase assay of Pto forms individually mutated at autophosphorylation sites. The effect of mutations at autophosphorylation sites on Pto autophosphorylation activity (A), and Pto phosphorylation of a kinase-deficient Pti1(K96N) (B), were tested in kinase assays *in vitro*. Pto mutants and Pti1(K96N) were expressed in bacteria as GST fusions. Kinase activity was tested in reactions containing the combinations of fusion proteins indicated in the figure. Proteins were fractionated by 10% SDS-PAGE and analyzed with a phosphorimager. The phosphorimager exposure (top panel) shows the proteins phosphorylated in each assay, whereas the Coomassie-stained gel (bottom panel) shows the protein species present in the reaction mixtures.

kinase-deficient form of the serine/threonine kinase Pti1 [GST-Pti1(K96N); Figure 5B] and the transcription factor Pti4 (data not shown). Similar results were also observed for the mutant Pto(T38D) (data not shown), suggesting that a negative charge at position 38 in the Pto amino acid sequence is not sufficient to restore the kinase activity of the molecule. All the other mutants, including Pto(S198A), that failed to elicit an HR *in vivo*, were still able to autophosphorylate and to phosphorylate both Pti1 and Pti4. These findings suggest that Thr38, which is the main Pto autophosphorylation site *in vitro*, is required for Pto autophosphorylation and for phosphorylation of Pto substrates. Since Pto kinase activity is required for the induction of an HR, the impaired kinase activity of Pto(T38A) was probably the source of its failure to elicit an HR *in vivo*.

Pto physical interactions with AvrPto, Pti1 and Pti4 are altered by mutations at certain Pto autophosphorylation sites

Physical interactions between Pto and additional proteins involved in speck disease resistance signaling have been characterized extensively using the yeast two-hybrid system (Gu and Martin, 1998). First, the AvrPto-Pto interaction is required for pathogen recognition and for the elicitation of the HR. Secondly, the Pto-Pti1 interaction is

Fig. 6. Yeast two-hybrid interactions of Pto autophosphorylation sites mutants with AvrPto, Pti1 and Pti4. **(A)** Expression of bait fusion proteins in the yeast strain EGY48. Wild-type and mutant forms of Pto were expressed in yeast as LexA fusions in the bait plasmid pEG202. LexA fusions were detected by chemiluminescent visualization in Western blots using polyclonal antibodies raised against LexA. In each lane, the Pto form expressed in the yeast strain EGY48 is indicated. **(B)** Protein–protein interactions quantified by β -galactosidase assays. EGY48 yeast cells contained Pto or a Pto mutant in the bait plasmid (pEG202), and AvrPto, Pti1 or Pti4 in the prey plasmid (pJG4-5), as indicated. Yeast strains were grown in galactose medium, lacking uracil, histidine and tryptophan, and tested for *lacZ* reporter gene activation. Bars represent β -galactosidase activity expressed in relative units (Reynolds and Lundblad, 1989). Data are the means of activities observed for four independent transformants $\pm$ SE. For each bait and prey combination, the assay was repeated at least three times with similar results.

proposed to be involved in the activation of Pti1 by Pto, as a step toward the elicitation of the HR. Finally, the Pto–Pti4 interaction is thought to mediate transcriptional activation of basic-type pathogenesis-related genes. To test the role of Pto autophosphorylation sites in protein–protein interactions, Pto forms mutated at autophosphorylation sites were cloned into the bait vector pEG202, as LexA fusion proteins. Bait vectors were transformed into EGY48 yeast cells, which expressed either AvrPto, Pti1 or Pti4 in the prey plasmid pJG4-5. All bait proteins were expressed in yeast at similar levels, as assessed by Western blot analysis using antibodies raised against the LexA fusion (Figure 6A). We assayed the interaction between the different protein combinations by monitoring activation of the reporter genes *lacZ* and *LEU2*. As shown in Figure 6B, a mutation at Thr38 abolished the interaction of Pto with all the interactors tested. This was in agreement with our previous findings that Pto(T38A) did not elicit an HR *in vivo*, lost its autophosphorylation activity and was not able to phosphorylate Pti1 and Pti4. It is interesting to note that the introduction of an aspartate at position 38 had the same effect on Pto interactions as the substitution of Thr38 with an alanine (data not shown). This suggests that a negative charge at this position is not sufficient for the interaction of Pto with additional components of the pathway. The

introduction into Pto of an alanine in place of Ser198, which *in vivo* interfered with the elicitation of the HR, did not effect AvrPto–Pto interaction, but significantly enhanced the interaction of Pto with Pti1. Mutations at autophosphorylation sites Thr199 and Thr288 resulted in a reduction in AvrPto–Pto and Pto–Pti1 interactions, correlating with a decrease in the ability of these mutants to induce an HR in the plant, as described above. Two additional mutants, Pto(T190A) and Pto(T195A), showed a normal interaction with AvrPto and a slightly weakened interaction with Pti1, as compared with wild-type Pto. However, the reduction in the interaction of these mutants with Pti1 was slight and did not appear to interfere with the elicitation of the HR. The interaction between Pto and the transcription factor Pti4 was significantly reduced by mutations at several autophosphorylation sites, i.e. Thr38, Thr190, Thr195, Ser198, Thr199 and Thr288, suggesting that the phosphorylation state of Pto is a key determinant for this interaction. However, all the mutants, with the exception of Thr38, still showed a significant interaction with Pti4 that was sufficient for Pto to phosphorylate Pti4 (data not shown). The biological effect of these mutations on the induction of pathogenesis-related genes, which are the proposed target of Pti4, awaits further analysis in stable transgenic plants expressing the mutant Pto forms in a background free of a functional Pto.

Fig. 7. Yeast two-hybrid interactions of Pto(S198A) with Pto interactors (Pti). EGY48 yeast cells contained Pto or Pto(S198A) in the bait plasmid pEG202, and Pti3, Pti7, Pti8, Pti9 or Pti10 in the prey plasmid pJG4-5, as indicated. Yeast strains were grown on galactose medium with X-gal lacking uracil, histidine and tryptophan, and tested for *lacZ* reporter gene activation. The plates were incubated at 30°C for 3 days and photographed.

Pto(S198A)* shows an impaired interaction with *Pti3* and *Pti10

To identify additional molecular mechanisms that are possibly involved in the elicitation of the HR and require Pto Ser198, we tested the physical interaction between the mutant Pto(S198A) and a group of Pto-interacting proteins (Pti; Zhou *et al.*, 1998). The Pto interactors tested were isolated previously by screening a tomato cDNA library using Pto as a bait in a yeast two-hybrid system (Zhou *et al.*, 1995). Among them were Pti3, which shows similarity at the amino acid level to the ATP-binding cassette (ABC), and Pti7, Pti8, Pti9 and Pti10, with no similarities to any proteins in the databases. We assayed in the yeast two-hybrid system the interactions between Pto(S198A) and the different Ptis, and compared them with the interactions between wild-type Pto and the same proteins. As shown in Figure 7, Pto(S198A) was not able to interact with Pti10, and showed a reduced interaction with Pti3. These results establish an intriguing correlation between the inability of Pto mutated at autophosphorylation site Ser198 to elicit an HR and its impaired physical interactions with Pti3 and Pti10. In addition, they point to Pti3 and Pti10 as putative components of a signaling pathway that, after activation by Pto, leads eventually to the hypersensitive response.

Discussion

We investigated the role of Pto autophosphorylation in molecular mechanisms that lead to speck disease resistance in tomato. We first identified sites autophosphorylated by Pto *in vitro*, and then tested the involvement of each site in molecular functions of the Pto kinase. These included the AvrPto–Pto-mediated elicitation of the hypersensitive response, Pto autophosphorylation activity, phosphorylation of substrates and protein–protein interactions. This strategy allowed us to concentrate our investigation on specific autophosphorylation sites, and to make a clear distinction between Pto autophosphorylation and phosphorylation activity. Our experimental approach was based on the observation that in several instances, sites phosphorylated *in vitro* by autophosphorylation or cross-phosphorylation mechanisms are also phosphorylated *in vivo* (e.g. Colwill *et al.*, 1996; Fish

Gietzen and Virshup, 1999). However, it is still possible that in the plant not all the sites autophosphorylated *in vitro* are functional, or conversely that additional sites are autophosphorylated.

Multiple autophosphorylation sites for multiple functions

By the extensive biochemical analysis of a tryptic digest of Pto autophosphorylated *in vitro*, we identified eight sites of autophosphorylation. These sites are scattered in distinct functional domains of the Pto molecule, as observed in a three-dimensional model obtained by comparison of Pto with protein kinases of known structure (Figure 8). The predicted Pto molecule resembles the typical structure of a protein kinase catalytic domain, consisting of a small lobe, which is involved in ATP binding and orientation, and a large lobe, which provides sites for substrate recognition and catalysis (Morgan and De Bondt, 1994). The small lobe represents the N-terminus of the molecule and consists predominantly of β -strands, while the large lobe comprises the C-terminus of the molecule and includes several α -helices. Pto autophosphorylation sites Ser17 and Thr38 are located outside the kinase catalytic domain in close proximity to the Pto small lobe and to the ATP-binding site. The lack of homology at the N-terminus of Pto with proteins that have been crystallized did not allow us to predict the spatial location of these sites with respect to the Pto catalytic domain. Since Pto autophosphorylation occurs via an intramolecular mechanism (Sessa *et al.*, 1998), it will be interesting to determine the complete Pto three-dimensional structure and to learn how the molecule folds so that Ser17 and Thr38 become accessible to the catalytic core of the kinase domain.

Six additional autophosphorylation sites were found in the Pto kinase catalytic domain, located in the large lobe of the molecule. Remarkably, sites Thr190, Thr195, Ser198 and Thr199 are within the Pto region corresponding to a particular segment, defined as the kinase activation domain, which in many protein kinases is phosphorylated and involved in the regulation of kinase activity (Johnson *et al.*, 1996). An additional phosphorylation site, Thr288, is located at the C-terminus of Pto outside the portion of the molecule whose structure was predicted by homology modeling.

Fig. 8. Location of Pto autophosphorylation sites in the predicted structure of Pto. The three-dimensional structure of the Pto kinase catalytic domain from residue 42 to 240 was predicted with the Swiss-model program (Guex and Peitsch, 1997; <http://www.expasy.ch/swissmod/SWISS-MODEL.html>), by using the crystal structure of the following proteins as templates: the kinase c-src (2src), the lymphocyte kinase (3lck) and the fibroblast growth factor receptor 1 in complex with AMP (2fgia), with the inhibitor SU5402 (1fgib), or the inhibitor SU4984 (1agwb); in parentheses are their accession codes in the Swiss-3D image collection database (Peitsch *et al.*, 1995; <http://www.expasy.ch/sw3d>). The location of autophosphorylation sites is indicated by white circles on the molecule backbone. Thr38, Ser17 and Thr288, which are not in the region included in the predicted structure, are shown outside the molecule. The N- and C-termini of the structure are indicated by the letters N and C, respectively. Arrows indicate the boundaries of the Pto activation domain, which lies between amino acids Asp182 and Glu211. β -strands and α -helices are colored in blue and red, respectively.

The distribution of autophosphorylation sites in the Pto molecule suggests that different sites may be involved in distinct mechanisms mediated by the Pto kinase during the onset of disease resistance. Autophosphorylation may affect the recognition specificity and physical interaction between Pto and AvrPto, or the activation of Pto by binding to AvrPto. In addition, it may be involved in interaction and phosphorylation of Pto downstream effectors, or represent a structural requirement for the proper molecular configuration. Multiple autophosphorylation events have been observed previously in receptor tyrosine kinases (Kashishian *et al.*, 1992; van der Geer and Hunter, 1993). Phosphorylated tyrosine residues were found to mediate interactions with different downstream signaling molecules, providing a mechanism that allows one receptor to initiate multiple signaling pathways. We can envisage a similar scenario for Pto, in which different autophosphorylation events mediate distinct responses to pathogen invasion, including elicitation of the HR, accumulation of PR proteins and the production of reactive oxygen species. Mutational analysis of Pto autophosphorylation sites allowed us to start investigating their possible functions, and provided evidence that indeed different sites participate in distinct molecular mechanisms.

Requirement for autophosphorylation at Thr38 for the elicitation of the HR

Thr38 is the primary site of Pto autophosphorylation *in vitro*. In addition, it was found by mutational analysis to be required for the elicitation of the HR, and for the interaction of Pto with AvrPto, Pti1 and Pti4. Moreover, Pto forms that are mutated at Thr38 lost their kinase activity, which has been shown in several instances to be required for the development of an HR (Scofield *et al.*, 1996; Tang *et al.*, 1996). This evidence suggests that autophosphorylation at Thr38 may lead to a conformational change in the structure of the molecule that allows Pto to autophosphorylate at additional sites and to interact with AvrPto, Pti1 and Pti4. This possibility is supported by the analysis of several mutations at two residues, Lys69 and Asp164, which are invariant amino acids in all protein kinases, and are required for ATP binding and catalysis, respectively (Hanks and Quinn, 1991). A substitution of Lys69 with a glutamine renders Pto kinase deficient and impairs its interaction with Pti1 (Zhou *et al.*, 1995). In addition, mutations of Lys69 to asparagine or glutamine, and of Asp164 to alanine, glutamate or glycine, abolished Pto kinase activity and the ability of Pto to interact with AvrPto (Scofield *et al.*, 1996; Tang *et al.*, 1996; Rathjen *et al.*, 1999). In this context, the only exception is represented by the substitution of Asp164 with asparagine, which disrupted Pto kinase activity but not the AvrPto–Pto interaction (Rathjen *et al.*, 1999). It is possible that the structure of Pto(D164N) mimics the conformation of autophosphorylated Pto. Taken together, these results strongly suggest that autophosphorylation at Thr38 is a critical step for Pto in gaining competence for binding to AvrPto, Pti1 and Pti4.

The evidence that mutation of Thr38 to aspartate has the same effect as an alanine substitution at that position indicates that a negative charge is not enough to restore Thr38 function. A three-dimensional structure of the Pto molecule will be required to understand how autophosphorylation of Thr38 and mutations at this site might affect the conformation of Pto.

A threonine corresponding to Pto Thr38 is conserved outside the catalytic domain of several Pto-homologous protein kinases from different organisms. These include the product of a susceptible *Pto* allele and the Fen protein kinase from tomato, the RLK Xa21 from rice, the IRAK kinase from mammals and the Pelle kinase from *Drosophila* (Table II). An intriguing parallel has been drawn between Pto, IRAK and Pelle kinases, based on their sequence similarity and involvement in defense pathways in the respective organisms (O'Neill and Greene, 1998). It will be interesting to test the functional conservation among these proteins and, in this context, to determine whether threonine residues, corresponding in IRAK and Pelle to Pto Thr38, are also major autophosphorylation sites required for their function.

Autophosphorylation in the Pto activation domain and the elicitation of the HR

The finding that four residues, Thr190, Thr195, Ser198 and Thr199, are autophosphorylated in the Pto activation domain adds more evidence for the central role of this region in Pto function. The Pto activation domain was first shown to be involved in the AvrPto–Pto physical inter-

Fig. 9. A model for the AvrPto–Pto signal transduction pathway leading to the hypersensitive response. The model is described in detail in the Discussion.

action (Scofield *et al.*, 1996; Tang *et al.*, 1996). It was then found that Thr204 in this domain is responsible for AvrPto recognition specificity (Frederick *et al.*, 1998). Although the function of Thr204 was suggested to be mediated by its phosphorylation, we did not find evidence indicating that this residue is a target *in vitro* for Pto autophosphorylation. The C-terminal part of the Pto activation domain was shown recently to be involved in the negative regulation of Pto kinase function (Rathjen *et al.*, 1999). In this region, mutations at Thr204 and Tyr207 to aspartate conferred to Pto the ability to induce an HR in the absence of AvrPto. The Pto gain-of-function mutants were still dependent on the functionality of the Prf protein, which is required for resistance to *P.syringae* pv. tomato and sensitivity to the insecticide fenthion (Salmeron *et al.*, 1996).

Here we showed that the Pto autophosphorylation site Ser198, in the Pto activation domain, is required for the elicitation of the HR and dispensable for the AvrPto–Pto physical interaction. This suggests that the molecular mechanism in which Ser198 is involved lies downstream of AvrPto recognition by Pto. The activation domain is the target of regulatory autophosphorylation in several serine/threonine and tyrosine kinases (Johnson *et al.*, 1996). This observation raises the possibility that autophosphorylation at Ser198 might induce Pto kinase activity as part of the Pto mechanism of activation by the AvrPto–Pto interaction. However, a regulatory function of kinase activity for Ser198 appears to be unlikely, since the mutant form Pto(S198A) is still able to phosphorylate Pti1 and Pti4 *in vitro* with an efficiency similar to wild-type Pto.

An alternative explanation for the requirement for Ser198 in the elicitation of the HR is that this residue is critical for the interactions between Pto and its downstream effectors. In line with this hypothesis, a mutation at Ser198 was found to alter the physical interaction of Pto with the Pto interactors Pti1, Pti3 and Pti10. The mutant Pto(S198A) showed an interaction with the serine/threonine kinase Pti1 that was 3-fold stronger than that of Pto. A high affinity Pto(S198A)–Pti1 complex could sequester Pto and Pti1, preventing them from functioning in the development of the HR. In addition, the same Pto(S198A) mutant had a reduced interaction with the ABC transporter Pti3, and with a protein of unknown function, Pti10 (Zhou *et al.*, 1998). The intriguing correlation between the inability of Pto(S198A) to elicit the HR and the reduced interaction with Pti3 and Pti10 suggests a function for Pti3 and Pti10 downstream of Pto in the signaling pathway that leads to the elicitation of the HR.

It is interesting to note that Ser198 is not conserved in a *Pto* allele from susceptible tomato plants (Jia *et al.*, 1997). In the translation product of this allele, the absence of a residue essential for the induction of the HR by Pto may account for its inability to confer disease resistance. However, Ser198 is conserved in the kinase activation domain of the rice RLK Xa21, which confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Song *et al.*, 1995). It will be interesting to test the role of this residue in Xa21-mediated disease resistance.

Thr199, which is an additional autophosphorylated residue of the kinase activation domain and lies adjacent

to Ser198, appears to participate in the elicitation of the HR, although it is not strictly required for this process. The proximity of Thr199 to Ser198, which is required for the HR, raises the possibility that the phenotype observed for Pto(T199A) is caused by a steric effect that partially compromises the function of Ser198. However, differences in the physical interaction with AvrPto and Pti1 observed for Pto(T199A) and Pto(S198A) suggest that these two residues affect the HR through different mechanisms. Reduced interactions with AvrPto and Pti1 may be the source of the weaker HR observed for Pto(T199A). Similarly to Pto(T199A), the mutant Pto(T288A) showed a weaker HR and reduced interactions with AvrPto and Pti1. This suggests that although they are 89 residues apart in the amino acid sequence, Thr199 and Thr288 may affect a similar mechanism involved in Pto function.

A model for the AvrPto–Pto-mediated elicitation of the HR

Based on previously reported evidence and the data presented in this report, we propose the following model for the elicitation of the HR mediated by the AvrPto–Pto interaction (Figure 9). Early after translation, a spontaneous event of intramolecular autophosphorylation at Thr38 stabilizes Pto in a structural conformation that is competent for autophosphorylation of additional sites and for binding to AvrPto. In tomato cells at rest, the Pto level is probably kept very low by a strict control mechanism to avoid activation of defense mechanisms in the absence of pathogen. This is in line with the evidence that over-expression of Pto in the absence of AvrPto activates defense responses and the development of an HR (Tang *et al.*, 1999). It remains to be tested whether Pto autophosphorylation is part of a control mechanism that is involved in Pto degradation, as observed for the Pto homolog IRAK in human cells (Yamin and Miller, 1997).

During pathogenesis, AvrPto is delivered by *P. syringae* directly into the plant cell, probably by a type III secretion system (Galan and Collmer, 1999), and it is recognized specifically by Pto through determinants located in the Pto activation domain. In this step, Thr204 appears to be of central importance in determining recognition specificity for AvrPto (Frederick *et al.*, 1998). The binding of AvrPto to Pto causes a conformational change in the structure of the Pto activation domain, which results in induction of Pto activity. In fact, the mutant Pto(Y207D), which has a substitution in a residue of the activation domain, mimics an active Pto (Rathjen *et al.*, 1999). Pto(Y207D) is able to induce an HR only in the presence of a functional Prf, whose role in the Pto signaling pathway is still unknown. It is not clear what the direct effect is of the conformational change in the Pto molecule, which is triggered by the interaction with AvrPto. It is possible that as a consequence of AvrPto binding, the catalytic core of Pto is now accessible to substrate(s), or that certain molecular domains, which were not available previously, are now exposed to autophosphorylation or to phosphorylation by an additional kinase. Alternatively, residues that in the absence of AvrPto were buried in the molecule, are now exposed and available for the physical interaction with substrate(s).

Active Pto is then able to initiate a signal transduction pathway leading to the HR by the interaction with downstream effectors and their phosphorylation. The Pto–Pti1 interaction is proposed to lead to phosphorylation of Pti1 in its activation domain at Thr233, and to be an initial step downstream of Pto toward the elicitation of the HR (Sessa *et al.*, 2000). Pto Ser198, possibly in its autophosphorylated form, is required for the elicitation of the HR and appears to participate in molecular mechanism(s) involving protein–protein interactions. A mutation at this residue increases the binding affinity of Pto for Pti1, while it reduces the interaction of Pto with Pti3 and Pti10. However, the function of Pti3 and Pti10 in speck disease resistance and the significance of their interactions with Pto await further investigation.

This model does not include signaling pathways originating from Pto that lead to defense responses in addition to the HR and contribute to the onset of disease resistance. The identification of Pto autophosphorylation sites and their mutants represent a valuable tool for future investigation of the involvement of Pto autophosphorylation also in these pathways.

Materials and methods

Site-directed mutagenesis

Mutagenesis of Pto autophosphorylation sites was performed in the plasmid pGEX-KG, containing a GST–Pto fusion protein (Sessa *et al.*, 1998), or in the shuttle vector pTEX containing the Pto-coding region under the control of the CaMV 35S promoter (Frederick *et al.*, 1998). Site-specific mutations were introduced into Pto using the Quickchange™ kit from Stratagene, according to the manufacturer's protocols. Sequences of oligonucleotides used for mutagenesis are available upon request from the corresponding author. The presence in the gene of the desired mutations was confirmed by nucleic acid sequencing, using an ABI Prism™ Genetic analyzer (Perkin Elmer).

Expression of fusion proteins and in vitro kinase assays

Pto, its mutagenized forms and Pti1(K96N) were expressed in bacteria as fusion proteins in-frame with the C-terminus of GST, and purified as described previously (Sessa *et al.*, 1998). Assays to test autophosphorylation of Pto mutants, and their phosphorylation of Pti1(K96N) and Pti4, were performed with GST–Pto forms immobilized on glutathione–agarose beads and the other components dissolved in solution as described (Sessa *et al.*, 1998). Large-scale phosphorylation reactions of 250 µg of GST–Pto or GST–Pto(T288A) were performed in 300 µl of reaction buffer [50 mM Tris–HCl pH 7.0, 1 mM dithiothreitol (DTT), 10 mM MnCl₂ and 20 µM ATP], containing 10 µCi of [γ -³²P]ATP (6000 Ci/mmol; Amersham Corp.).

HPLC mapping, purification and mass spectrometry analysis of phosphopeptides

Trypsin digests of autophosphorylated GST–Pto or GST–Pto(T288A) fusions for HPLC fractionation were prepared as described (Sessa *et al.*, 2000). Digests were resuspended in 0.1% trifluoroacetic acid (TFA) and resolved by reverse-phase HPLC with a Vydac C18 (250/2.1 mm) column at a flow rate of 0.15 ml/min. The column was developed with a linear acetonitrile gradient (0.75%/min for 60 min and 2.75%/min for 20 min) in 0.1% TFA. The eluant was monitored by UV absorbance at 214 nm, while fractions were collected at 30 s intervals, and subjected to Cerenkov counting. Radioactive fractions corresponding to GST–Pto phosphopeptides A–I (Figure 1) were HPLC-purified further by using the same column and a slow acetonitrile gradient (0.3%/min for 50 min) in 0.1% TFA.

Partially purified phosphopeptides were then subjected to MALDI-TOF/MS analysis performed at the Biotechnology Resource Laboratory of the W.M. Keck Facility of Yale University, New Haven, CT, using a research grade, VG Tofspec SE time-of-flight (TOF) mass spectrometer (Micromass, Manchester, UK), equipped with a delayed extraction ion source and operating in the reflectron mode. Sample preparation was as previously described (Sessa *et al.*, 2000). The accuracy of mass

assignment was within $\pm 0.05\%$ using external calibration. The MS-digest computer program provided by the UCSF Mass Spectrometry Facility was used to calculate the average masses of all possible peptide and phosphopeptide fragments from GST-Pto, and the m/z value of the mass spectral peaks for the corresponding MH^+ ions (<http://prospector.ucsf.edu/ucsfhtml3.2/msdigest.htm>).

Manual Edman degradation and phosphoamino acid analysis

HPLC-purified phosphopeptides were covalently linked to an arylamine-Sequelon disk using the Sequelon™-AA Reagent kit and protocols (Millipore). Manual degradation of immobilized phosphopeptides was then performed in sequential cycles, as described by Sullivan and Wong (1991). The amount of radioactivity released by each degradation cycle was measured by Cerenkov counting. Phosphoamino acid analysis was performed as described by van der Geer *et al.* (1993).

Yeast two-hybrid interactions

To subclone the Pto forms mutated at autophosphorylation sites into the bait plasmid pEG202, an *EcoRI* site was first inserted by site-directed mutagenesis upstream of their coding regions in the pTEX shuttle vector. Mutagenesis was performed as described above using the following oligonucleotides: 5'-GAGGACAGGGTACCGAATTCATGGGAAGC-AAG-3' and 5'-CTTGCTTCCCATGAATTCGGTACCCTGTCCTC-3'. Pto mutants were excised from the pTEX vector by using *EcoRI* and *BamHI* restriction enzymes, and inserted at the corresponding sites in the pEG202 vector, in-frame with the LexA-coding region. Two-hybrid interactions were tested, using standard protocols (Golemis *et al.*, 1995), in the yeast strain EGY48, transformed sequentially with the *lacZ* reporter plasmid, the bait plasmid expressing a LexA-Pto fusion and the prey plasmid pJG4-5, expressing either AvrPto, Pti1 or Pti4 (Zhou *et al.*, 1995, 1997; Tang *et al.*, 1996). Quantitative assays of β -galactosidase activity in liquid cultures were performed as described (Reynolds and Lundblad, 1989), except that yeast cells were grown in a minimal liquid medium containing 10% galactose, and lacking uracil, histidine and tryptophan. Expression in yeast for all LexA fusion proteins was determined by Western blot analysis, by using LexA antibodies and chemiluminescent visualization (ECL kit; Amersham), as previously described (Frederick *et al.*, 1998).

Agrobacterium-mediated transient assay in *N.benthamiana*

Site-directed mutagenesis of Pto autophosphorylation sites was performed in the pTEX shuttle vector containing Pto driven by the CaMV 35S promoter, and followed by the Nos terminator (Frederick *et al.*, 1998). The expression cassettes were excised by the *EcoRI* restriction enzyme and inserted at the *EcoRI* site between the T-DNA right and left borders of the binary vector pBTEx or pBTEx::avrPto, which contains the coding region of *avrPto* under the control of the CaMV 35S promoter and Nos terminator (Frederick *et al.*, 1998). The pBTEx plasmids were introduced into *A.tumefaciens* strain EHA105 by electroporation. *Agrobacterium* strains were grown for ~10 h at room temperature in induction medium [50 mM MES pH 5.6, 0.5% (w/v) glucose, 1.7 mM NaH_2PO_4 , 20 mM NH_4Cl , 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$ and 200 μM acetosyringone]. Bacterial cultures were then diluted to an $OD_{600} = 0.4$, and infiltrated into leaves of 6- to 8-week-old *N.benthamiana* plants. The HR developed within 3–5 days, and the severity of the symptoms was recorded with scores from 1 to 5 (where 1 = no symptoms and 5 = cell death in the whole infiltrated area). In a typical experiment, three plants were infiltrated in 5–8 different leaves with *Agrobacterium* strains expressing the following combinations of proteins: (i) the Pto mutant to be tested and AvrPto; (ii) wild-type Pto and AvrPto, as a positive control; and (iii) AvrPto, as a negative control. An HR index reflecting the degree of symptoms developed by each Pto mutant, as compared with wild-type Pto, was calculated according to the following formula: $[\Sigma(PtoM + AvrPto) - \Sigma(AvrPto)] / [\Sigma(Pto + AvrPto) - \Sigma(AvrPto)]$. In this formula, the entries within the brackets are the scores for symptoms caused by the expression of the indicated proteins, and PtoM represents the specific mutant tested.

Acknowledgements

We thank Y.Barron and M.Ercolano for technical assistance; A.J.Bogdanove for helpful comments on the manuscript; Y.Gu for providing recombinant Pti4; A.M.Mahrenholz and M.Bower for helpful suggestions regarding HPLC analysis; and K.L.Stone at the W.M.Keck Facility of Yale University, New Haven, CT for assistance with MALDI-

TOF/MS analysis. This research was supported, in part, by Postdoctoral Award No. FI-248-97 from BARD, the United States-Israel Binational Agricultural Research and Development Fund (G.S.), United States Department of Agriculture grant NRI-9901355, National Science Foundation grant MCB-9630635 and a David and Lucile Packard Foundation Fellowship.

References

- Baker, B., Zambryski, P., Staskiewicz, B. and Dinesh-Kumar, S.P. (1997) Signaling in plant-microbe interactions. *Science*, **276**, 726–733.
- Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C. and Duncan, P.I. (1996) The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. *EMBO J.*, **15**, 265–275.
- Fish Gietzen, K. and Virshup, D.M. (1999) Identification of inhibitory autophosphorylation sites in casein kinase I ϵ . *J. Biol. Chem.*, **274**, 32063–32070.
- Frederick, R.D., Thilmony, R.L., Sessa, G. and Martin, G.B. (1998) Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase. *Mol. Cell*, **2**, 241–245.
- Galan, J.E. and Collmer, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*, **284**, 1322–1328.
- Golemis, E.A., Gyuris, J. and Brent, R. (1995) Interaction trap/two-hybrid system to identify interacting proteins. In Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds), *Current Protocols in Molecular Biology*. John Wiley and Sons Inc., New York, NY, Vol. 3, pp. 20.1.1–20.1.28.
- Gu, Y.-Q. and Martin, G.B. (1998) Molecular mechanisms involved in bacterial speck disease resistance of tomato. *Philos. Trans. R. Soc. Lond. B*, **353**, 1455–1461.
- Gu, Y.-Q., Yang, C., Tara, V.K., Zhou, J. and Martin, G.B. (2000) *Pti4* is induced by ethylene and salicylic acid and its product is phosphorylated by the Pto kinase. *Plant Cell*, in press.
- Guex, N. and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-Pdbviewer: an environment for comparative protein modelling. *Electrophoresis*, **18**, 2714–2723.
- Hanks, S.K. and Quinn, M. (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol.*, **200**, 38–62.
- Hammond-Kosack, K.E. and Jones, J.D.G. (1996) Resistance gene-dependent plant defense responses. *Plant Cell*, **8**, 1773–1791.
- Hammond-Kosack, K.E. and Jones, J.D.G. (1997) Plant disease resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**, 575–607.
- Jia, Y., Loh, Y.-T., Zhou, J. and Martin, G.B. (1997) Alleles of *Pto* and *Fen* occur in bacterial speck-susceptible and fenthion-insensitive tomato cultivars and encode active protein kinases. *Plant Cell*, **9**, 61–73.
- Johnson, L.N., Noble, M.E.M. and Owen, D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell*, **85**, 149–158.
- Kashishian, A., Kazlauskas, A. and Cooper, J.A. (1992) Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase *in vivo* [published erratum appears in *EMBO J.*, 1992, **11**, 3809]. *EMBO J.*, **11**, 1373–1382.
- Loh, Y.-T. and Martin, G.B. (1995) The *Pto* bacterial resistance gene and the *Fen* insecticide sensitivity gene encode functional protein kinases with serine/threonine specificity. *Plant Physiol.*, **108**, 1735–1739.
- Martin, G.B. (1999) Functional analysis of plant disease resistance genes and their downstream effectors. *Curr. Opin. Plant Biol.*, **2**, 273–279.
- Martin, G.B., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganai, M.W., Spivey, R., Wu, T., Earle, E.D. and Tanksley, S.D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*, **262**, 1432–1436.
- Morgan, D.O. and De Bondt, H.L. (1994) Protein kinase regulation: insights from crystal structure analysis. *Curr. Opin. Cell Biol.*, **6**, 239–246.
- O'Neill, L.A.J. and Greene, C. (1998) Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J. Leukoc. Biol.*, **63**, 650–657.
- Peitsch, M.C., Wells, T.N., Stampf, D.R. and Sussman, J.L. (1995) The Swiss-3D image collection and PDB-Browser on the World-Wide Web. *Trends Biochem. Sci.*, **20**, 82–84.
- Rathjen, J.P., Chang, J.H., Staskiewicz, B.J. and Michelmore, R.W. (1999) Constitutively active *Pto* induces a *Prf*-dependent hypersensitive response in the absence of *avrPto*. *EMBO J.*, **18**, 3232–3240.

- Reynolds, A. and Lundblad, V. (1989) Yeast vectors and assay for expression of cloned genes. In Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds), *Current Protocols in Molecular Biology*. John Wiley and Sons Inc., New York, NY, Vol. 2, pp. 13.6.1–13.6.4.
- Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H. and Jones, J.D.G. (1999) Rapid *Avr9*- and *Cf-9*-dependent activation of MAP kinases in tobacco cell cultures and leaves; convergence of resistance gene, elicitor, wound, and salicylate responses. *Plant Cell*, **11**, 273–287.
- Salmeron, J.M., Oldroyd, G.E.D., Rommens, C.M.T., Scofield, S.R., Kim, H.-S., Lavelle, D.T., Dahlbeck, D. and Staskawicz, B.J. (1996) Tomato *Prf* is a member of the leucine-rich-repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell*, **86**, 123–133.
- Scofield, S.R., Tobias, C.M., Rathjen, J., Chang, J.H., Lavelle, D.T., Michelmore, R.W. and Staskawicz, B.J. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science*, **274**, 2063–2065.
- Sessa, G. and Martin, G.B. (2000) Protein kinases in the plant defense response. In Callow, J.A., Kreis, M. and Walker, J.C. (eds), *Advances in Botanical Research*. Academic Press, London, UK, vol. 32, pp. 379–404.
- Sessa, G., D'Ascenzo, M., Loh, Y.-T. and Martin, G.B. (1998) Biochemical properties of two protein kinases involved in disease resistance signaling in tomato. *J. Biol. Chem.*, **273**, 15860–15865.
- Sessa, G., D'Ascenzo, M. and Martin, G.B. (2000) The major site of the Pti1 kinase phosphorylated by the Pto kinase is located in the activation domain and is required for Pto–Pti1 physical interaction. *Eur. J. Biochem.*, **267**, 171–178.
- Shen, B. and Manley, J.L. (1998) Phosphorylation modulates direct interactions between the Toll receptor, Pelle kinase and Tube. *Development*, **125**, 4719–4728.
- Song, W.-Y. et al. (1995) A receptor kinase-like protein encoded by the rice disease resistance gene *Xa21*. *Science*, **270**, 1804–1806.
- Stone, J.M., Collinge, M.A., Smith, R.D., Horn, M.A. and Walker, J.C. (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase. *Science*, **266**, 793–795.
- Sullivan, S. and Wong, T.W. (1991) A manual sequencing method for identification of phosphorylated amino acids in phosphopeptides. *Anal. Biochem.*, **197**, 65–68.
- Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y. and Martin, G.B. (1996) Initiation of plant disease resistance by physical interaction of *AvrPto* and *Pto* kinase. *Science*, **274**, 2060–2063.
- Tang, X., Xie, M., Kim, Y.J., Zhou, J., Klessig, D.F. and Martin, G.B. (1999) Overexpression of *Pto* activates defense responses and confers broad resistance. *Plant Cell*, **11**, 15–29.
- van der Geer, P. and Hunter, T. (1993) Mutation of Tyr697, a GRB-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. *EMBO J.*, **12**, 5161–5172.
- van der Geer, P., Luo, K., Sefton, B.M. and Hunter, T. (1993) Phosphopeptide mapping and phosphoamino acid analysis on cellulose thin-layer plates. In Hardie, D.G. (ed.), *Protein Phosphorylation: A Practical Approach*. IRL Press at Oxford University Press, Oxford, UK, pp. 31–59.
- Yamin, T.T. and Miller, D.K. (1997) The interleukin-1 receptor-associated kinase is degraded by proteasomes following its phosphorylation. *J. Biol. Chem.*, **272**, 21540–21547.
- Zhang, S. and Klessig, D.F. (1998) Resistance gene *N*-mediated *de novo* synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. *Proc. Natl Acad. Sci. USA*, **95**, 7433–7438.
- Zhou, J., Loh, Y.-T., Bressan, R.A. and Martin, G.B. (1995) The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by *Pto* and is involved in the plant hypersensitive response. *Cell*, **83**, 925–935.
- Zhou, J., Tang, X. and Martin, G.B. (1997) The *Pto* kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. *EMBO J.*, **16**, 3207–3218.
- Zhou, J., Tang, X., Frederick, R. and Martin, G.B. (1998) Pathogen recognition and signal transduction by the *Pto* kinase. *J. Plant Res.*, **111**, 353–356.

Received January 26, 2000; revised and accepted March 20, 2000