The Tomato NBARC-LRR Protein Prf Interacts with Pto Kinase in Vivo to Regulate Specific Plant Immunity

Tatiana S. Mucyn,a Alfonso Clemente,b,1 Vasilios M.E. Andriotis,a,2 Alexi L. Balmuth,a Giles E.D. Oldroydb,2 Brian J. Staskawicz,b and John P. Rathjena,3

a The Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom
b Department of Plant and Microbial Biology, University of California, Berkeley, California 94720

Immunity in tomato (Solanum lycopersicum) to Pseudomonas syringae bacteria expressing the effector proteins AvrPto and AvrPtoB requires both Pto kinase and the NBARC-LRR (for nucleotide binding domain shared by Apaf-1, certain R gene products, and CED-4 fused to C-terminal leucine-rich repeats) protein Prf. Pto plays a direct role in effector recognition within the host cytoplasm, but the role of Prf is unknown. We show that Pto and Prf are coincident in the signal transduction pathway that controls ligand-independent signaling. Pto and Prf associate in a coregulatory interaction that requires Pto kinase activity and N-myristoylation for signaling. Pto interacts with a unique Prf N-terminal domain outside of the NBARC-LRR domain and resides in a high molecular weight recognition complex dependent on the presence of Prf. In this complex, both Pto and Prf contribute to specific recognition of AvrPtoB. The data suggest that the role of Pto is confined to the regulation of Prf and that the bacterial effectors have evolved to target this coregulatory molecular switch.

INTRODUCTION

Plants possess highly effective innate immune systems. Recognition and response to potential pathogens is encoded at two levels. First, microbial components called PAMPs (for pathogen-associated molecular patterns) are ligands for extracellular receptor proteins that activate signaling pathways leading to immunity (Chinchilla et al., 2006). Second, pathogen secretes effector proteins that can be recognized by host molecules encoded by plant resistance (R) genes. These mechanisms have been referred to as PAMP-triggered immunity and effector-triggered immunity, respectively (Chisholm et al., 2006). Effector-triggered immunity relies on R gene–specific recognition of pathogen components derived from avirulence (Avr) genes. The specific R–Avr interaction is also referred to as the gene-for-gene effect. For plant pathogenic bacteria, Avr proteins are generally a subset of effector proteins that are delivered to the host cytoplasm via a specialized type III secretion apparatus (Bonas and Van den Ackerveken, 1997). Effector proteins normally contribute to bacterial fitness via the suppression of host immunity (Chang et al., 2004), but they trigger strong defense responses in the presence of cognate R proteins.

R proteins confer recognition of diverse effectors, and in some cases the same R protein is responsible for the recognition of unrelated effectors (Rathjen and Moffett, 2003). The largest class of plant R genes encodes a conserved NBARC (for nucleotide binding domain shared by Apaf-1, certain R gene products, and CED-4) ATPase domain fused to C-terminal leucine-rich repeats (NBARC-LRR proteins), sometimes juxtaposed with other structural elements such as an N-terminal TIR domain (Dangl and Jones, 2001). Important related animal proteins include the CATERPILLER/NOD/NALP proteins, which contain a related NACHT-type ATPase domain and control innate immunity (Ting and Williams, 2005), and the Apaf-1 and CED-4 proteins, which contain a homologous NBARC ATPase domain and regulate apoptotic cell death (Cecconi, 1999). A key question for both animal and plant proteins involved in innate immunity is the mode of activation by elicitor molecules. The mammalian NOD1 and NOD2 proteins recognize, respectively, 3-d-glutamyl-meso-diaminopimelic acid and muramyl dipeptide derived from the bacterial cell wall component peptidoglycan, but direct interaction between these ligands and NODs has not been shown (Strober et al., 2006). For plant R proteins, both direct and indirect interactions with Avr proteins have been described. The best understood example is the activation of the Arabidopsis thaliana NBARC-LRR RPS2 by the cognate bacterial effector protein AvrRpt2. AvrRpt2 cleaves the cofactor RIN4, leading to the derepression of RPS2 signaling (Axtell and Staskawicz, 2003; Mackey et al., 2003). Similarly, the Arabidopsis NBARC-LRR RPS5 requires the cofactor PBS1 kinase for activity (Shao et al., 2003). However, these examples do not provide a general model for Avr-R recognition, because examples of direct interaction also exist (Jia et al., 2000; Deslandes et al., 2003). Thus, the mechanisms by which plant R proteins recognize and keep pace with the evolution of pathogen Avr components are of major
biological interest. In addition, little is known of the steps involved in the activation of NBARC-LRR proteins.

The unrelated effector proteins AvrPto and AvrPtoB of *Pseudomonas syringae pv tomato* (*Pst*) trigger disease resistance on tomato (*Solanum lycopersicum*) cultivars containing the *Pto* gene. *Pto*, a protein kinase (Pedley and Martin, 2003), interacts directly with each effector through overlapping surface areas (Wu et al., 2004). *Pto* requires the NBARC-LRR protein Prf for function (Salmeron et al., 1996), although how Prf contributes to *Pto*-mediated resistance is unknown. However, *Pto* and Prf are clustered at a single genomic locus with four *pto* homologs, which suggests cooperative function (Chang et al., 2002). *Pto* appears to signal by a conformational change rather than by phosphorylation of a downstream substrate(s), but the proximal events in signaling are obscure (Wu et al., 2004). Prf lies downstream or coincident with *Pto* in the signal transduction pathway (Rathjen et al., 1999), but the epistatic relationship between these genes has not been resolved further. In addition to gene-for-gene resistance conferred by *Pto* (Rathjen et al., 1999), but the epistatic relationship between *Pto* and Prf is underpinned by constitutive physical interaction (Chang et al., 2002). *Pto* requires the NBARC-LRR protein Prf for signaling (Rommens et al., 1995). Individual expression of *Pto* or Prf Is a Signaling Protein Regulated by *Pto*

In contrast with the data described above, we found that extreme overexpression of *Pto* from a dexamethasone (DEX)-inducible promoter (Dex:Prf) in *N. benthamiana* leaves resulted in a *Pto*-independent HR within 24 h of induction (Figure 2A). This *Pto*-independent phenotype was dependent on *Nb* Sgl1 and *Nb* Hsp90 (data not shown), consistent with the activation of a disease resistance pathway. To exclude a role for *N. benthamiana* *pto* homologs in this HR, we amplified three of them from *Nb* genomic DNA (*Nb* *Pth1* to *Pth3*) and silenced them using virus-induced gene silencing (VIGS) in wild-type *N. benthamiana* (Ratcliff et al., 2001). Expression of *AvrPto* fused to green fluorescent protein (3SS:avrPto-GFP) induces Nb Prf-dependent cell death in *N. benthamiana* that is independent of tomato *Pto* (J.S. de Vries and J.P. Rathjen, unpublished data). Silencing of *Nb* *Pth1* suppressed the HR induced by transient expression of *AvrPto*-GFP, confirming the successful silencing of *Nb* *Pth1*. The Dex:Prf HR was not compromised in *N. benthamiana* plants silenced with empty vector (TRV:EV) or *Nb* *Pth1* constructs (Figure 2B). Similar data were obtained for *Nb* *Pth2* and *Nb* *Pth3* (data not shown). Therefore, the Dex:Prf HR was independent of *Pto* or a closely related homolog. These data are important because *Pto* or its constitutive gain-of-function

**RESULTS**

**Pto and Prf Act Coincidentally in Signal Transduction**

We previously showed that Prf acts coincident with or downstream of *Pto* (Rathjen et al., 1999). To further define this relationship, we investigated a requirement for *Pto* in the CDR phenotype of the transgenic tomato line I2 (*Pto*/Pto; *Prf*-I2/*Prf*-I2), which overexpresses Prf from its native promoter (Oldroyd and Staskawicz, 1998). We crossed the mutant allele *pto-11* (Salmeron et al., 1994) into the I2 line to create the homozygous line I2/pto-11 (pto-11/pto-11; *Prf*-I2/*Prf*-I2). The growth of *Pst* strain T1 lacking avrPto was similar on I2/pto-11 plants to that on RioGrande 76R plants (*Pto*/Pto; *Prf*/*Prf*), whereas growth on I2 plants was reduced by ~1000-fold (Figure 1A), demonstrating that *Pto* is required for the CDR phenotype. To investigate this further, we expressed Prf and *Pto* in leaves of *Nicotiana benthamiana*, a Solanaceous relative of tomato that supports *Pto* signaling (Rommens et al., 1995). Individual expression of *Pto* or *Pto* from the strong 3SS promoter did not induce visible defense responses (Figure 1B). By contrast, coexpression of 3SS:Prf in the presence but not the absence of 3SS:*Pto* led to cell death, indicative of the hypersensitive response (HR), within 4 d (Figure 1B). The *Pto*–Prf ligand-independent HR was weaker and slower than that caused by coexpression of AvrPto and *Pto* in *N. benthamiana*, which occurred after 36 to 48 h (Rathjen et al., 1999). This ligand-independent phenotype was not dependent on *Pto* overexpression, because coexpression of 3SS:Prf with *Pto* expressed from its native promoter (*Prf*:1C:*Pto*) also induced the HR (Figure 1B). These results corroborate the observation that transgenic tomato overexpressing Prf required *Pto* for the CDR phenotype. To further analyze the role of *Pto* in the cell death phenotype, 3SS:Prf was coexpressed with the mutants *ptoD164N* and *ptoG2A*, which are deficient in kinase activity and N-myristoylation, respectively (de Vries et al., 2006), and do not induce the AvrPto-dependent HR. Coexpression of 3SS:Prf with 3SS:*ptoD164N* or 3SS:*ptoG2A* failed to induce cell death (Figure 1C). Therefore, the ligand-independent HR in *N. benthamiana* caused by Prf overexpression was subject to typical requirements for ligand-dependent *Pto* signaling. Xiao et al. (2003) described the *ptoG50S* mutant, which abolished ligand-independent but not AvrPto-dependent signaling in transgenic tomato overexpressing *Pto*. The *ptoG50S* mutant retained AvrPto-dependent signaling when expressed transiently in *N. benthamiana* (see Supplemental Figure 1 online), whereas its coexpression with 3SS:Prf did not induce the HR (Figure 1C). Protein gel blotting confirmed that the lack of HR was not attributable to impaired protein accumulation. These results suggest that the *N. benthamiana* cell death phenotype is a manifestation of the ligand-independent signaling phenomenon observed in tomato. Overall, the data are consistent with *Pto* and Prf acting coincidentally in the signal transduction pathway.

**Pto and Prf Coregulate Plant Immunity**

We previously showed that Prf acts coincident with or downstream of *Pto* (Rathjen et al., 1999). To further define this relationship, we investigated a requirement for *Pto* in the CDR phenotype of the transgenic tomato line I2 (*Pto*/Pto; *Prf*-I2/*Prf*-I2), which overexpresses Prf from its native promoter (Oldroyd and Staskawicz, 1998). We crossed the mutant allele *pto-11* (Salmeron et al., 1994) into the I2 line to create the homozygous line I2/pto-11 (pto-11/pto-11; *Prf*-I2/*Prf*-I2). The growth of *Pst* strain T1 lacking avrPto was similar on I2/pto-11 plants to that on RioGrande 76R plants (*Pto*/Pto; *Prf*/*Prf*), whereas growth on I2 plants was reduced by ~1000-fold (Figure 1A), demonstrating that *Pto* is required for the CDR phenotype. To investigate this further, we expressed Prf and *Pto* in leaves of *Nicotiana benthamiana*, a Solanaceous relative of tomato that supports *Pto* signaling (Rommens et al., 1995). Individual expression of *Pto* or

([53x479]independent phenotypes can be seen in the absence of AvrPto

The unrelated effector proteins AvrPto and AvrPtoB of *Pseudomonas syringae pv tomato* (*Pst*) trigger disease resistance on tomato (*Solanum lycopersicum*) cultivars containing the *Pto* gene. *Pto*, a protein kinase (Pedley and Martin, 2003), interacts directly with each effector through overlapping surface areas (Wu et al., 2004). *Pto* requires the NBARC-LRR protein Prf for function (Salmeron et al., 1996), although how Prf contributes to *Pto*-mediated resistance is unknown. However, *Pto* and Prf are clustered at a single genomic locus with four *pto* homologs, which suggests cooperative function (Chang et al., 2002). *Pto* appears to signal by a conformational change rather than by phosphorylation of a downstream substrate(s), but the proximal events in signaling are obscure (Wu et al., 2004). Prf lies downstream or coincident with *Pto* in the signal transduction pathway (Rathjen et al., 1999), but the epistatic relationship between these genes has not been resolved further. In addition to gene-for-gene resistance conferred by *Pto* (Rathjen et al., 1999), but the epistatic relationship between *Pto* and Prf is underpinned by constitutive physical interaction (Chang et al., 2002). *Pto* requires the NBARC-LRR protein Prf for signaling (Rommens et al., 1995). Individual expression of *Pto* or Prf Is a Signaling Protein Regulated by *Pto*

In contrast with the data described above, we found that extreme overexpression of *Pto* from a dexamethasone (DEX)-inducible promoter (Dex:Prf) in *N. benthamiana* leaves resulted in a *Pto*-independent HR within 24 h of induction (Figure 2A). This *Pto*-independent phenotype was dependent on *Nb* Sgl1 and *Nb* Hsp90 (data not shown), consistent with the activation of a disease resistance pathway. To exclude a role for *N. benthamiana* *pto* homologs in this HR, we amplified three of them from *Nb* genomic DNA (*Nb* *Pth1* to *Pth3*) and silenced them using virus-induced gene silencing (VIGS) in wild-type *N. benthamiana* (Ratcliff et al., 2001). Expression of *AvrPto* fused to green fluorescent protein (3SS:avrPto-GFP) induces Nb Prf-dependent cell death in *N. benthamiana* that is independent of tomato *Pto* (J.S. de Vries and J.P. Rathjen, unpublished data). Silencing of *Nb* *Pth1* suppressed the HR induced by transient expression of *AvrPto*-GFP, confirming the successful silencing of *Nb* *Pth1*. The Dex:Prf HR was not compromised in *N. benthamiana* plants silenced with empty vector (TRV:EV) or *Nb* *Pth1* constructs (Figure 2B). Similar data were obtained for *Nb* *Pth2* and *Nb* *Pth3* (data not shown). Therefore, the Dex:Prf HR was independent of *Pto* or a closely related homolog. These data are important because *Pto* or its constitutive gain-of-function
variants are completely inactive in the absence of Prf (Rathjen et al., 1999; Mysore et al., 2002; Wu et al., 2004). Therefore, although Pto and Prf act coincidentally, Prf encodes the functions necessary for downstream signaling. However, we cannot completely exclude the existence of a N. benthamiana protein kinase that complemented the Dex:Prf overexpression phenotype but was incapable of AvrPto-GFP recognition and insufficiently similar to Nb Pth1 to be silenced in the VIGS experiment.

Strikingly, the Dex:Prf HR could be suppressed by coexpression with either ptoG2A or ptoD164N but not when coexpressed with ptoG50S or wild-type Pto (Figure 2C). Loss of the HR was not attributable to the lack of Prf protein (Figure 2C). Therefore, although the Dex:Prf HR was independent of Pto, it was dominantly suppressed by inactive pto mutants. These data uncover an additional role for Pto in the negative regulation of Prf upstream of Pto kinase activity and confirm the coincident action of the proteins. The switch between the negative and positive regulatory states of Pto is presumably triggered by its kinase activity.

Figure 1. Pto and Prf Are Coincident in a Signal Transduction Pathway Leading to Disease Resistance.

(A) Requirement of Pto for constitutive disease resistance in tomato mediated by Prf overexpression. Left panel, growth of Pst strain T1 on tomato genotypes as indicated. The values are means of three samples, from three different plants, treated equivalently in the same experiment. Error bars indicate sd. The graph presented is representative of three replicate experiments. Right panel, RNA gel blot showing Prf expression. The 18S rRNA band is shown as a loading control.

(B) Codependence of Prf and Pto for the overexpression HR in N. benthamiana. Genes expressed from the cauliflower mosaic virus 35S and ProPto promoters are indicated, and EV indicates the empty binary vector control. The photograph was taken 3.5 d after infiltration.

(C) The Prf overexpression HR requires normal Pto signaling capabilities. Left panel, 35S:Prf was coexpressed with wild-type Pto, the kinase-knockout mutant ptoD164N, the N-myristoylation mutant ptoG2A, or the weak kinase variant ptoG50S. The photograph was taken 5 d after infiltration. Right panel, protein accumulation was confirmed by protein gel blot using the anti-HA antibody for Prf-3HA detection (top gel) or polyclonal anti-Pto antiserum (middle gel). wt indicates untransformed tissue, and other constructs are as indicated. Coomassie blue (CBB) staining of the protein gel blot membrane confirmed equivalent protein loading (bottom gel).

Pto and Prf Contribute Reciprocally to Protein Accumulation in N. benthamiana

We tested whether the differential requirement for Pto in the cell death phenotypes induced by 35S:Prf and Dex:Prf was related to the levels of expression from these promoters. Typically, Prf accumulation from 35S:Prf transient expression was equivalent to or less than that found in wild-type tomato extracts (Figure 3A). Coexpression of 35S:Pto led to much higher Prf accumulation, whether Pto was expressed transiently or as a 35S:Pto stable transgene in N. benthamiana line 38-12 (Rommens et al., 1995) (see also Figure 1C). Higher levels of Prf protein accumulated
from Dex:Prf than from 35S:Prf expression. Thus, the pto-independent cell death induced by Dex:Prf expression is attributable to exceptionally high Prf levels. To test whether Prf contributes to Pto accumulation, we expressed Pto transiently from its native promoter in N. benthamiana in the presence or absence of Prf (Figure 3B). In these experiments, Pto accumulated very poorly in the absence of tomato Prf, whereas qualitatively more protein was visible when tomato Prf was coexpressed from its native promoter. The highest level of Pto accumulation was seen when Prf was coexpressed from the 35S promoter. Overall, the data show that Pto and Prf contribute reciprocally to protein accumulation in N. benthamiana.

Prf Interacts with Pto in Vivo

The data presented above suggest that Pto and Prf interact in vivo. To test this, the genes encoding these proteins were coexpressed transiently in N. benthamiana leaves under the control of their respective native promoters. Pto was expressed as a fusion protein with three C-terminal hemagglutinin (HA) epitopes and a single FLAG epitope (Pto-3HAF), whereas Prf was expressed as a fusion to three C-terminal HA epitopes (Prf-3HA). The ligand-independent HR was not observed under these conditions. Protein gel blot analysis demonstrated that both proteins were expressed at almost undetectable levels in the crude extract (Figure 4A). Protein extracts from infiltrated leaves were subjected to immunoprecipitation with anti-FLAG beads. Pto was enriched in the bead fraction, indicating successful immunoprecipitation. Prf was detectable in the bead fraction when Pto-3HAF was present in the extract, but it was absent when the extract lacked Pto-3HAF. These data demonstrate that Pto and Prf interact in vivo. To confirm this interaction using a reverse coimmunoprecipitation strategy, Pto was expressed from its native promoter as a fusion with triple C-terminal HA epitopes (Pto-3HA), whereas the Prf protein was expressed from its native promoter as a fusion with five C-terminal c-myc epitopes (Prf-5myc). Protein extracts from infiltrated leaves were immunoprecipitated with anti-myc beads to capture Prf-5myc. Pto was detectable in the bead fraction only when Prf was present, but not when the extract lacked Prf (Figure 4A). To test this interaction in tomato, a stable transgenic line carrying a single copy of ProPto::Pto-FLAG was generated (line P27; see Supplemental Figure 2 online). Protein extracts from P27 and a control line transformed with an empty binary vector (line P21)
were immunoprecipitated with anti-FLAG beads (Figure 4B). Specific elution of Pto from the beads with FLAG peptide resulted in the release of Prf from P27 (Pto-FLAG) but not P21 (EV) extracts, indicating specific interaction between Pto and Prf in tomato. Therefore, the interaction between Pto and Prf observed in N. benthamiana was not an artifact of transient expression. Overall, the data demonstrate that Pto and Prf form a constitutive protein complex in vivo.

AvrPto is believed to interact directly with Pto in vivo (Pedley and Martin, 2003). To assess whether AvrPto disrupts the Pto–Prf association, ProPto: Pto-3HAF and ProPrf:Prf-5myc were coexpressed in the stable transgenic N. benthamiana plants expressing 35S:Pto (38-12). Note the low level of Pto accumulation in these plants. Proteins were detected specifically as indicated, and equal protein loading was confirmed by Coomassie Brilliant Blue (CBB) staining of the protein gel blot membrane. AvrPto did not cause dissociation of the Pto–Prf complex. However, we were unable to identify a trimolecular complex containing AvrPto, Pto, and Prf in these experiments.

Several important Pto mutants that influence Prf-dependent signaling negatively (G2A, D164N, and G50S) or positively (L205D) (Wu et al., 2004) have been described (see above). We tested the ability of these mutants to interact with Prf by coimmunoprecipitation. 35S:Prf-5myc was coexpressed transiently with ProPto: Pto-3HAF or each mutated gene, or with an EV.

---

**Figure 3.** Pto and Prf Contribute Reciprocally to Protein Accumulation.

(A) Pto contributes to Prf accumulation in planta. Prf was detected from protein extracts of RioGrande 76R or prf3 tomato or from wild-type and 35S:Pto transgenic N. benthamiana plants (Rommens et al., 1995). Left, extracts of tomato containing (76R) or lacking (prf3) a functional Prf gene. Middle, expression of 35S:Prf or Dex:Prf in wild-type N. benthamiana, with (35S:Pto) or without (EV) Pto as indicated. For 35S:Prf expression, numbers refer to days after infiltration with Agrobacterium, whereas for Dex:Prf, numbers refer to hours after induction with DEX. Right, expression of 35S:Prf in stable transgenic N. benthamiana plants expressing 35S:Pto (38-12). Note the low level of Pto accumulation in these plants. Proteins were detected specifically as indicated, and equal protein loading was confirmed by Coomassie Brilliant Blue (CBB) staining of the protein gel blot membrane.

(B) Prf contributes to Pto accumulation. ProPrf: Pto-3HAF was coexpressed with EV, ProPrf:Prf-5myc, or 35S:Prf-5myc in N. benthamiana leaves. Tissues were harvested at 2.5 d after infiltration. Equal amounts of protein derived from crude extracts of infiltrated tissues were loaded on SDS-PAGE gels for immunoblotting. Pto and Prf were detected using antisera as indicated. The asterisk indicates a nonspecific cross-reacting band.
Figure 4. Prf Interacts with Pto in Vivo.

(A) Prf and Pto coimmunoprecipitate from *N. benthamiana* extracts. Left, *N. benthamiana* leaves were transiently transformed with ProPto:Pto-HAF, ProPrf:Prf-3HA, or a 1:1 mixture of these strains. Strains for individual gene expression were balanced with *A. tumefaciens* containing an empty vector construct. Infiltrated leaves were harvested 3 d after infiltration for extraction of proteins, and protein extracts were subjected to immunoprecipitation as indicated. Equal amounts by volume of crude extract (Input) and supernatant after immunoprecipitation (Unbound) were loaded onto the SDS-PAGE gel for immunoblotting. The bead fractions (Beads) were ∼80 times more concentrated than the crude extract. Right, *N. benthamiana* leaves transiently transformed as above with ProPto:Pto-3HA, ProPrf:Prf-5myc, or a 1:1 mixture of these strains. Samples were prepared as above, except for the use of anti-myc agarose beads for immunoprecipitation. The bead fractions were ∼20 times more concentrated than the crude extract. Prf-5myc was undetectable in the crude extract. The asterisk indicates a cross-reacting band. Equal protein loading was confirmed by Coomassie blue (CBB) staining of the protein gel blot membrane.

(B) Prf coimmunoprecipitates with Pto in tomato extracts. Protein extracts from transgenic tomato lines expressing ProPto:Pto-FLAG (P27) or EV (P21) were subjected to immunoprecipitation using anti-FLAG beads before specific elution of bound proteins with the FLAG peptide (Elution).

(C) The Pto–Prf interaction is not disrupted by AvrPto. Transgenic Dex:avrPto-HA *N. benthamiana* plants were transiently transformed with ProPto:Pto-3HAF, ProPrf:Prf-3HA, or a 1:1 mixture of these strains as above. Transformed leaves were treated with DEX 2.5 d after infiltration to induce AvrPto expression. Tissues were harvested before treatment or 6 h after treatment with DEX. Protein extracts were immunoprecipitated with anti-FLAG beads followed by immunoblotting with anti-HA antibody to detect Prf-3HA, AvrPto-HA, and Pto-3HAF. The bead fractions were ∼80 times more concentrated than the crude extract in this experiment. The asterisk indicates the heavy chain of the anti-FLAG antibody released from the beads.

(D) Interaction of Prf with key *pto* mutants. *N. benthamiana* plants were first transiently transformed with 35S:Prf-5myc, then infiltrated 24 h later with EV, ProPto:ptoD164N-3HAF, ProPto:ptoG2A-3HAF, ProPto:ptoG50S-3HAF, or ProPto:ptoL205D-3HAF. Tissues were harvested 2 d after the second infiltration. Protein extracts were subjected to immunoprecipitation using anti-FLAG beads before elution of bound proteins with the FLAG peptide. Equivalent amounts of crude extract before immunoprecipitation (Input) or the final eluates (Elution) were loaded onto SDS-PAGE gels for immunoblotting. Pto and Prf were detected using specific antisera as indicated. The asterisk indicates a cross-reacting band.
control, in *N. benthamiana*. Protein gel blot analysis of the total protein fraction extracted from transformed leaves showed that only *pto*G2A and *pto*L205D were detectable in the total protein extract, whereas Prf accumulation was easily detected and less variable. Immunoprecipitation of each extract with anti-FLAG beads followed by elution with FLAG peptide revealed the presence of Prf in all treatments, except where EV was expressed instead of Pto. Similar to the total extract, the *pto*G2A and *pto*L205D proteins were more abundant in the elution fraction than the other Pto forms. *pto*G2AS accumulated very poorly. The amount of Prf in the bead fractions was roughly proportional to the other Pto forms. *pto*G50S control, in *N. benthamiana*.

Protein gel blot analyses revealed elution of Prf primarily in the fractions 8 to 10 mL after the column void volume (Figure 5A). This corresponds to a molecular weight of ~400,000 to 600,000, greater than the predicted Prf size of ~200,000. The observed peak corresponds to Prf, because no corresponding band was seen in similar protein gel blots derived from RioGrande 76R prf3 mutants (*pto*/Pto; prf3/prf3), which lack a functional Prf gene (Salmeron et al., 1996). Pto could not be detected in these experiments because of its extremely low level of accumulation in tomato. To examine the presence of Prf within the apparent Prf complex, we expressed both Prf and Pto transiently in *N. benthamiana*. In these experiments, it was necessary to over-express both genes from the 35S promoter to enable detection of the proteins. Comparison of the Prf profile from these extracts after SEC revealed a similar pattern to that derived from wild-type tomato extracts in the presence (Figure 5B) or absence of tomato Pto (data not shown). Probing of the same fractions for Pto revealed its presence in two peaks. The predominant peak (L) corresponded to the molecular weight of monomeric (uncomplexed) Pto. The secondary peak (H) entirely overlapped the Prf elution peak in the ~400,00 to 600,000 region. Strikingly, Pto in the H fraction migrated as a doublet on SDS-PAGE, with the upper band more prominent than the lower band (Figure 5C). This is suggestive of autoprophosphorylation, because it was removed by dephosphorylation and was not present in a kinase-deficient mutant, *pto*D164N (Figure 5D). Second, silencing of endogenous *Nb Prf* in the absence of tomato Prf severely reduced the amount of Pto in the H fraction (Figure 5E), demonstrating that Pto requires Prf for both autoprophosphorylation and its presence in the upper molecular weight range. These data are consistent with a signaling complex that contains Prf and Pto before stimulation by cognate Avr proteins.

**Pto Resides in a High Molecular Weight Complex Dependent on the Presence of Prf**

The data described above show that Pto and Prf interact. To explore whether they exist in a molecular complex, we prepared total protein extracts from RioGrande 76R tomato leaves and fractionated them by size-exclusion chromatography (SEC). Protein gel blot analyses revealed elution of Prf primarily in the fractions 8 to 10 mL after the column void volume (Figure 5A). This corresponds to a molecular weight of ~400,000 to 600,000, greater than the predicted Prf size of ~200,000. The observed peak corresponds to Prf, because no corresponding band was seen in similar protein gel blots derived from RioGrande 76R prf3 plants (*pto*/Pto; prf3/prf3), which lack a functional Prf gene (Salmeron et al., 1996). Pto could not be detected in these experiments because of its extremely low level of accumulation in tomato. To examine the presence of Prf within the apparent Prf complex, we expressed both Prf and Pto transiently in *N. benthamiana*. In these experiments, it was necessary to over-express both genes from the 35S promoter to enable detection of the proteins. Comparison of the Prf profile from these extracts after SEC revealed a similar pattern to that derived from wild-type tomato extracts in the presence (Figure 5B) or absence of tomato Pto (data not shown). Probing of the same fractions for Pto revealed its presence in two peaks. The predominant peak (L) corresponded to the molecular weight of monomeric (uncomplexed) Pto. The secondary peak (H) entirely overlapped the Prf elution peak in the ~400,00 to 600,000 region. Strikingly, Pto in the H fraction migrated as a doublet on SDS-PAGE, with the upper band more prominent than the lower band (Figure 5C). This is suggestive of autoprophosphorylation, because it was removed by dephosphorylation and was not present in a kinase-deficient mutant, *pto*D164N (Figure 5D). Second, silencing of endogenous *Nb Prf* in the absence of tomato Prf severely reduced the amount of Pto in the H fraction (Figure 5E), demonstrating that Pto requires Prf for both autoprophosphorylation and its presence in the upper molecular weight range. These data are consistent with a signaling complex that contains Prf and Pto before stimulation by cognate Avr proteins.

**The N Terminus of Prf Interacts with Pto**

The C-terminal half of Prf corresponds to a canonical R protein with coiled-coil (CC) and NBARC-LRR domains, but the N terminus is poorly characterized. We detected weak homology between Prf and other solanaceous R proteins (Mi, Sw-5, Hero, and R1) (Milligan et al., 1998; Brommonschenkel et al., 2000; Ballvora et al., 2002; Ernst et al., 2002) between amino acids 546 and 900, so this was arbitrarily named the Solanaceae domain (SD). Amino acids 1 to 546 did not show similarity to known proteins, so these were designated the N-term domain (Figure 6A). To identify a subdomain of Prf that interacts with Pto, we generated truncated versions of Prf corresponding to the isolated N-term, SD-CC-NBARC-LRR, or CC-NBARC-LRR domain. All truncated sequences were placed under the control of the 35S promoter as genetic fusions with a sequence encoding three C-terminal HA epitope tags and transiently expressed in 35S:Pto transgenic *N. benthamiana*. Immunoprecipitation of protein extracts derived from these experiments with anti-HA beads indicated that Pto interacted with the N-term domain of Prf but not with the SD-CC-NBARC-LRR or CC-NBARC-LRR domain (Figure 6B). A processing event corresponding to the loss of ~10,000 from the Prf N-term was evident in these experiments (Figures 6B and 6C). To confirm this interaction using a reverse coimmunoprecipitation strategy, N-term-3HA was expressed in wild-type *N. benthamiana* leaves in the presence or absence of 35S:Pto-FLAG. Protein extracts from infiltrated leaves were immunoprecipitated with anti-FLAG beads (Figure 6C). Full-length and processed N-term fragments were both detectable in the bead fraction only in the presence of Pto-FLAG. The smaller N-term fragment comigrated with the heavy chain of the anti-FLAG antibody used for immunoprecipitation. However, close examination of the protein gel blot confirmed that both the full-length and processed N-term fragments interacted with Pto.

**Prf Contributes to AvrPtoB Recognition in N. benthamiana**

Proximity between Prf and Pto suggests that Prf might participate in effector recognition. Tomato Pto complements *avrPto* recognition in *N. benthamiana*, leading to functional disease resistance, and this is dependent on Nb Prf (Rommens et al., 1995; Lu et al., 2003). Interestingly, tomato Pto does not confer *avrPto* recognition in *N. benthamiana* (Kim et al., 2002), suggesting that an additional host component is absent or dysfunctional in this species. Prf is an obvious candidate for this proposed molecule. To test this, we coexpressed 35S:*avrPtoB* and 35S:Prf in 35S:Pto transgenic *N. benthamiana* (Figure 7A). Expression of *avrPtoB* in these plants induced the HR only in the presence of tomato Pto. This HR appeared 2 d before the ligand-independent HR resulting from the coexpression of 35S:Pto and 35S:*Pto* (Figure 1B). Coexpression of 35S:Prf and 35S:*avrPtoB* in the absence of Pto did not result in the HR, indicating that both tomato Pto and Prf are required for *avrPtoB* recognition (Figure 7A). Coexpression of *Pro*Prf*avrPtoB* with 35S:*avrPtoB* did not result in a macroscopic HR in 35S:Pto transgenic plants (data not shown). However, microscopic cell death was greatly enhanced in the combined presence of Prf, Pto, and *avrPtoB* relative to the same experiment lacking *avrPtoB* (Figure 7B). Thus, ectopic
Figure 5. Prf and Pto Coelute in a High Molecular Weight Fraction during SEC.

(A) Elution of Prf by SEC–fast protein liquid chromatography (FPLC). Total protein extracts from wild-type 76R (Prf/Prf) (top panel) or prf3 (prf3/prf3) (bottom panel) tomato leaves were separated by SEC-FPLC before concentration of the fractions preceding SDS-PAGE and protein gel blotting with specific antisera as indicated. Numbers refer to the volume of elution (in mL) beyond the void volume of the column. The peaks of elution of the molecular weight standards are indicated.

(B) Elution of Prf and Pto by SEC-FPLC after transient expression in N. benthamiana. 35S:Prf was coexpressed with 35S:Pto-3HAF in leaves of N. benthamiana before separation by SEC-FPLC and protein gel blotting as described above. Top panel, elution of Prf; bottom panel, elution of Pto. Fractions 7 to 11 were designated peak H, and fractions 33 to 37 were designated peak L.

(C) Altered migration of Pto-3HAF from H (left) and L (right). The L fraction was diluted 1:30 before loading for comparison.

(D) Altered migration is a result of Pto autophosphorylation. Left panel, a protein extract from tissue coexpressing Pro Pto: Pto-HAF with 35S:Prf was subjected to immunoprecipitation using anti-FLAG beads before elution of Pto with the FLAG peptide. The eluate was treated with λ phosphatase (PPase) plus phosphatase inhibitor (left lane), λ phosphatase without subsequent incubation (middle lane), or λ phosphatase with incubation for 10 min (right lane). Right panel, extracts from tissues coexpressing Pro Pto: Pto-HAF or Pro Pto:ptoD164N-HAF with 35S:Prf were subjected to purification using anti-FLAG beads as above. Eluates were resolved on the same SDS gel and visualized by protein gel blotting.

(E) Pto requires Prf both for presence and phosphorylation in peak H. Left panel, equivalent loadings of peak H fractions derived from tissue expressing both 35S:Pto-3HAF and 35S:Prf (lanes 1), 35S:Pto-HAF only in leaves silenced for empty vector (lanes 2), or 35S:Pto-HAF in leaves silenced for Nb Prf (lanes 3). Middle panel, protein gel blot with anti-Pto antisera showing the accumulation of Pto in total extracts of the tissues described in the left panel. Right panel, Coomassie blue (CBB) staining of the protein gel blot membrane showing equivalent loading.
expression of tomato Prf allowed a level of AvrPtoB recognition by Pto in *N. benthamiana*.

**DISCUSSION**

The experiments presented here demonstrate that *Pto* and *Prf* are mutually required for ligand-independent pathway activation under moderate levels of gene expression. *Pto* overexpression in tomato plants constitutively activates *Prf*-dependent defense responses in the absence of AvrPto (Tang et al., 1999; Xiao et al., 2003). Similarly, overexpression of *Prf* leads to enhanced resistance to a number of normally virulent bacterial and viral pathogens (Oldroyd and Staskawicz, 1998). We show here that *Prf*-overexpressing tomato requires *Pto* for the CDR phenotype. The codependence of *Prf* and *Pto* for ligand-independent signaling in tomato indicates that they act coincidentally in this pathway. In *N. benthamiana*, a comparable phenotype resulted from the expression of 35S:*Prf* in the presence of *Pto*, regardless of the level of expression of *Pto*. Similarly, the native *Pto* gene was sufficient for the CDR phenotype in *Prf*-overexpressing tomato. The requirement for both *Pto* and *Prf* in the ligand-independent HR in *N. benthamiana* again illustrates that *Pto* and *Prf* act

---

**Figure 6.** The N-Terminal Domain of Prf Interacts with Pto.

(A) Subdomain diagram of the Prf protein. Numbers indicate amino acids in the derived protein sequence of Prf. N-term, N-terminal domain; SD, Solanaceae domain; CC, coiled-coil domain; NBARC, ATPase domain; LRR, leucine-rich repeat domain.

(B) Pto coimmunoprecipitates with the N-term domain of Prf. 35S:*Pto* transgenic *N. benthamiana* was infiltrated with *A. tumefaciens* strains expressing 35S:N-term-3xHA, 35S:CC-NBARC-LRR-3xHA, or 35S:SD-CC-NBARC-LRR-3xHA or not infiltrated, as indicated. Proteins were extracted from leaves harvested 3 d after infiltration and immunoprecipitated with anti-HA beads followed by SDS-PAGE and immunoblotting with anti-HA or anti-Pto antiserum. Equivalent amounts of protein from the crude extract (Input) and supernatant after immunoprecipitation (Unbound) were loaded. The bead fractions (Beads) were approximately nine times more concentrated than the crude extract.

(C) The Prf N-term domain coimmunoprecipitates with Pto. Wild-type *N. benthamiana* leaves were infiltrated with *A. tumefaciens* containing 35S:*Pto*-FLAG or 35S:N-term-3xHA, or a 1:1 mixture of both strains, as indicated. Protein extracts were obtained and treated as above except that immunoprecipitation was performed using FLAG beads. The bead fractions were ~10 times more concentrated than the crude extract. The asterisks indicate a cross-reacting band of ~50,000 that corresponds to the heavy chain of the antibody used for immunoprecipitation.
coincidentally and demonstrates that the Pto/Prf-dependent HR in N. benthamiana relates to the CDR phenotype observed in tomato. Pto required both kinase activity and N-myristoylation for the HR induced by 35S:Prf expression. Therefore, ligand-independent and -dependent signaling by Pto are mechanistically related. Further evidence that the tomato and N. benthamiana phenotypes are manifestations of the same signaling phenomenon comes from the behavior of ptoG50S, a mutant with weak kinase activity (V.M.E. Andriotis and J.P. Rathjen, unpublished data). ptoG50S was deficient in ligand-independent signaling in transgenic tomato but mediated AvrPto-specific immunity (Xiao et al., 2003). Similarly, ptoG50S did not induce a 35S:Prf-dependent HR in N. benthamiana, but the HR could be induced by further coexpression of AvrPto (see Supplemental Figure 1 online). Tang et al. (1999) proposed that Pto triggers basal defense responses through a low level of unregulated kinase activity. Our data are consistent with this view but extend the underlying model to show that constitutive signaling is a function of the interaction between Pto and Prf.

Prf rather than Pto appears to encode the functions necessary for the activation of downstream signaling. Extreme overexpression of Prf using a DEX-inducible promoter induced a HR that was independent of tomato Pto or N. benthamiana Pto homologs. Conversely, Pto is incapable of signaling in the absence of a functional Prf gene (Salmeron et al., 1994; Rathjen et al., 1999; Mysore et al., 2002). Thus, within the confidence provided by VIGS, although Pto and Prf act coincidentally in signal transduction, Prf acts as the transduction module.

Ligand-independent signaling by Pto–Prf in N. benthamiana was apparently also a function of increased Prf accumulation in the presence of Pto. In this way, Pto appears to contribute to the stability of Prf. Enhancement of Prf signaling also required Pto signaling capability, so the increase in Prf protein levels was not sufficient for the ligand-independent HR. There was no disparity in Prf accumulation between tomato lines possessing or lacking the Pto gene (see Supplemental Figure 3 online). However, susceptible tomato lines express several closely related pto homologs that may act as redundant factors for Prf stability (Chang et al., 2002). Pto also accumulated to higher levels in the presence of tomato Prf in N. benthamiana. Similar experiments were not possible in tomato because of a lack of suitably sensitive and specific antisera. Overall, it seems that coexpression of Pto and Prf contributes to the stability of each respective protein through an unknown mechanism(s) that probably includes protein–protein interaction.

The Dex:Prf HR was compromised by key pto mutants deficient in kinase activity or N-myristoylation. In these experiments, constitutive signaling by overexpressed Prf was possibly attributable to the accumulation of a small fraction that misfolds into the active conformation. The pto mutants apparently lock Prf into an inactive conformation and hence unveil a capacity for negative regulation of Prf by Pto. We previously identified a negative
regulatory patch on the surface of the Pto kinase domain; it is possible that negative regulation of Prf is mediated by this patch (Wu et al., 2004). Overall, the data are consistent with a precise switching role for Pto between the two states of suppression and activation of Prf, mediated by its kinase activity. The level of signaling through Pto–Prf resulting in either ligand-independent or -dependent immunity is presumably directly correlated with the kinase activity of Pto.

Regulation of Prf by Pto is underpinned by physical interaction. This was clearly demonstrated using a variety of communoprecipitation assays under conditions of native levels of gene expression. The interaction is constitutive, which explains the ligand-independent signaling phenotypes. In addition, the Pto–Prf interaction was not disrupted by the presence of AvrPto or by any of several mutations that altered Pto function. We have not yet found direct evidence of a tertiary complex composed of Prf, Pto, and AvrPto. One reason for this is that the Pto–Prf complex is unstable in the presence of nonionic detergents (T.S. Mucyn and J.P. Rathjen, unpublished data), conditions that are necessary for the release of AvrPto from the plasma membrane. In general, it has been very difficult across a variety of examples to show an association between Avr proteins and cognate resistance proteins or protein complexes (Belkhadir et al., 2004). Therefore, it is possible in principle that such interactions are characteristically weak or transient.

We showed previously that although Pto requires kinase activity for Avr-dependent activation, constitutive gain-of-function forms signal independently of kinase activity. Thus, the active form of Pto seems to be distinguished by a conformational change. Constitutive interaction between Pto and Prf suggests important roles for Prf in regulating and responding to this process. Inhibition of the Dex:Prf HR by a kinase-deficient mutant suggests that Pto negatively regulates Prf before the activation of kinase activity. Conversely, active forms of Pto require Prf for signaling, suggesting that Prf is responsible for interpretation of the active form of Pto. Therefore, Pto and Prf appear to form a molecular switch that is regulated at one level by Pto kinase activity. However, Pto kinase activity itself needs to be regulated (Wu et al., 2004), and some observations are consistent with a role for Prf in this capacity (see below). It was proposed previously that Pto activates a kinase cascade initiated by phosphorylation of the Ser/Thr protein kinase Pti1 and contributes to defense gene regulation by phosphorylation of the Pt4/5/6 transcription factor family (Zhou et al., 1995, 1997). Roles for Pti genes in ligand-dependent signaling were not supported by silencing them using antisense technology (Xiao et al., 2003). In addition, there is no detectable ligand-independent signaling by Pto leading to resistance phenotypes in the absence of Prf (Mysore et al., 2002; Xiao et al., 2003), which apparently rules out alternative modes of signaling for Pto. We believe that the Pto–Prf interaction explains the ligand-independent signaling by Pto leading to resistance phenotypes in the absence of Prf (Mysore et al., 2002; Xiao et al., 2003). In addition, there is no detectable ligand-independent signaling by Pto leading to resistance phenotypes in the absence of Prf (Mysore et al., 2002; Xiao et al., 2003), which apparently rules out alternative modes of signaling for Pto. We believe that the Pto–Prf interaction explains the ligand-independent and -dependent signaling. Therefore, the major role of Pto in immune signaling is the regulation of Prf.

Analysis of protein complexes containing Prf provides further insight into Pto–Prf coregulation. Native Prf from tomato extracts eluted from SEC in a resolved peak corresponding to an estimated size of ~400,000 to 600,000. This pattern of elution was larger than expected for the predicted size of Prf (~200,000) and could be reconstituted by transient expression of 35S:Prf in N. benthamiana. Pto was also overexpressed in these experiments to ensure its detection, which was impossible under conditions of native expression. SEC showed that the majority of Pto eluted in a fraction corresponding to the uncomplexed, monomeric form. However, a small amount coeluted with Prf, and several lines of evidence suggested that this fraction was complexed with Prf. Most importantly, Pto was almost completely lost from the higher molecular weight fraction after silencing of Nb Prf (Figure 5D). We also found that Pto in the higher molecular weight fraction was bandshifted relative to uncomplexed Pto. This bandshift was a result of Pto autophosphorylation, because it was absent when the kinase mutant ptoD164N was coexpressed with tomato Prf. Strikingly, it was also absent or diminished in the absence of tomato Prf. The bandshift is indicative of a Pto autophosphorylation event that seems to be promoted in the presence of tomato Prf, although we cannot yet distinguish whether it is related to complex maturation or active signaling. In addition, the bandshift occurs upstream of effector recognition, which may provide some insight into ligand-independent signaling by Pto–Prf. The apparent mass of the Pto–Prf complex is suggestive of the presence of other proteins, although we cannot exclude overestimation of size as a result of aberrant migration on the SEC column.

The region of Prf sufficient for interaction with Pto resides in the N-term domain, approximately between amino acids 100 and 550. This is nominally reminiscent of the interaction of Arabidopsis RIN4 with the N-terminal CC domain of RPM1 (Mackey et al., 2002), although the Prf N-term domain is a novel sequence of unknown function. Following the line of argument above, it seems likely that the N-term–Pto domain could provide a regulatory node in addition to the NBARC-LRR portion of Prf. Furthermore, we found evidence of a possible divergence in function between the tomato and N. benthamiana Prf proteins. Expression of Pto in N. benthamiana is sufficient for recognition of AvrPto but not AvrPtoB (Kim et al., 2002). Coexpression of tomato Prf in Pto transgenic N. benthamiana conferred a level of AvrPtoB recognition leading to cell death phenotypes. We suggest two possible explanations for this phenomenon. First, an indirect model, in which Prf does not participate in recognition but relays the signal emanating from the AvrPtoB–Pto interaction. In this scenario, Nb Prf would be less efficient than tomato Prf at relaying an AvrPtoB-generated signal. Second, a model in which Prf plays a direct role in AvrPtoB recognition, which is not complemented by Nb Prf. The clearest AvrPtoB-dependent cell death phenotype was obtained with Prf overexpression, which could be consistent with either model. An appropriate experiment to differentiate the models would be to express Nb Prf transiently from the 3SS promoter in Pto transgenic N. benthamiana, which would be expected to restore AvrPtoB-dependent cell death if the lack of recognition was attributable to weaker signal transduction.

Some other observations suggest that the indirect model is less likely. First, Nb Prf is clearly sufficient for AvrPto recognition. This shows that an effector-dependent signal mediated by Pto can be transferred efficiently to Nb Prf. Second, we found that transient expression of tomato Prf from its native promoter, which constitutes underexpression relative to Prf levels in tomato (cf. Figures 4A and 4C), specified AvrPtoB-dependent microscopic cell death in the presence of Pto. Therefore, the total level
of Prf in this experiment is increased only marginally over wild-type levels, potentially discounting the indirect model. The anti–cell death activity of AvrPtoB could explain why Prf overexpression was required to obtain the macroscopic HR (Abramovitch et al., 2003). Last, recognition of both AvrPto and AvrPtoB is mediated by an almost identical surface patch on Pto (Wu et al., 2004). This indicates that a second component required for AvrPtoB recognition is dysfunctional in N. benthamiana. Prf is an obvious candidate for such a molecule, although it is formally possible that an unknown component contributes. Proximity of Pto to the Prf N-term suggests that some Prf residues could contact AvrPtoB and consequently influence recognition, either positively or negatively. Conversely, AvrPto may be too small to contact Prf, or it may not contact residues that are polymorphic between the tomato and N. benthamiana Prf proteins. Clearly, further experimentation is necessary to resolve whether Prf plays a role in effector recognition. Potential effector interaction outside of the NBARC-LRR domain is surprising because the LRR subdomain of R proteins is often assumed to control effector recognition (Ellis et al., 2000; Mondragon-Palomino et al., 2002). However, our data do not exclude a role for the Prf NBARC-LRR domain in the recognition of effectors.

The broad conclusion of this work is that the Pto–Prf complex is a regulatory switch that controls immune signaling. Pto contributes to this regulation through a patch of residues that overlap the kinase catalytic cleft, jointly controlling kinase capability, interaction with effector proteins, and regulation of signaling (Wu et al., 2004). Conversely, the contribution of Prf to Pto regulation is unknown but probably includes control of Pto kinase activity. Thus, the interaction between Pto and Prf is likely to be highly coevolved at the molecular level. It follows that mutations within the presumed coregulatory interface of either protein could lead to inactivation or inappropriate activation of signaling, both of which would be detrimental to the host. This requirement for coevolution provides an explanation for the genomic colocation of Prf with the Pto gene family. Consistent with this idea, Chang et al. (2002) showed that expression of either of the Pto family members Fen or Pto3 in N. benthamiana leads to Nb Prf-dependent HR. How can this requirement for precise functionality be reconciled with an additional role as receptors of the cognate effector proteins? Phytopathogenic bacteria evolve a role in effector recognition. Potential effector interaction outside of the Prf N-term suggests that some Prf residues could contact AvrPtoB and consequently influence recognition, either positively or negatively. Conversely, AvrPto may be too small to contact Prf, or it may not contact residues that are polymorphic between the tomato and N. benthamiana Prf proteins. Clearly, further experimentation is necessary to resolve whether Prf plays a role in effector recognition. Potential effector interaction outside of the NBARC-LRR domain is surprising because the LRR subdomain of R proteins is often assumed to control effector recognition (Ellis et al., 2000; Mondragon-Palomino et al., 2002). However, our data do not exclude a role for the Prf NBARC-LRR domain in the recognition of effectors.

The broad conclusion of this work is that the Pto–Prf complex is a regulatory switch that controls immune signaling. Pto contributes to this regulation through a patch of residues that overlap the kinase catalytic cleft, jointly controlling kinase capability, interaction with effector proteins, and regulation of signaling (Wu et al., 2004). Conversely, the contribution of Prf to Pto regulation is unknown but probably includes control of Pto kinase activity. Thus, the interaction between Pto and Prf is likely to be highly coevolved at the molecular level. It follows that mutations within the presumed coregulatory interface of either protein could lead to inactivation or inappropriate activation of signaling, both of which would be detrimental to the host. This requirement for coevolution provides an explanation for the genomic colocation of Prf with the Pto gene family. Consistent with this idea, Chang et al. (2002) showed that expression of either of the Pto family members Fen or Pto3 in N. benthamiana leads to Nb Prf-dependent HR. How can this requirement for precise functionality be reconciled with an additional role as receptors of the cognate effector proteins? Phytopathogenic bacteria evolve a role in effector recognition. Potential effector interaction outside of the Prf N-term suggests that some Prf residues could contact AvrPtoB and consequently influence recognition, either positively or negatively. Conversely, AvrPto may be too small to contact Prf, or it may not contact residues that are polymorphic between the tomato and N. benthamiana Prf proteins. Clearly, further experimentation is necessary to resolve whether Prf plays a role in effector recognition. Potential effector interaction outside of the NBARC-LRR domain is surprising because the LRR subdomain of R proteins is often assumed to control effector recognition (Ellis et al., 2000; Mondragon-Palomino et al., 2002). However, our data do not exclude a role for the Prf NBARC-LRR domain in the recognition of effectors.

METHODS

Introduction of the pto-11 Allele into I2 Plants

The transgenic Prf-overexpressing line I2 (Oldroyd and Staskawicz, 1998) of tomato (Solanum lycopersicum) was crossed with the Pto mutant pto-11 (Salmeron et al., 1994). The F1 generation was self-fertilized, and plants homozygous for the pto-11 mutation and I2 transgene were selected in the F3 generation.

Pseudomonas syringae pv tomato Growth Assays

Growth curves were conducted as described previously (Garland and Staskawicz, 1993). Pst strain T1 was used at a concentration of 1 × 10⁴ colony-forming units/mL for growth curves.

Cloning and Expression of Prf

The entire Prf open reading frame from RioGrande 76S was subcloned from the cosmids pSOR2-7 (Salmeron et al., 1996) into pCB301 (Xiang et al., 1999) using the BamHI and SacI sites. The 5‘ end of the gene was modified by the removal of introns I and II upstream of the ATG translational start site and the removal of introns IV and V downstream of the TAG translational terminator. The Prf open reading frame was then subcloned into pTF540 (Rathjen et al., 1999) under the control of the cauliflower mosaic virus 35S promoter and fused to various epitope tags as described in the text. For expression from the genomic promoter, the 35S promoter was removed by digestion with EcoRI and replaced with an ~6-kb EcoRI fragment that restored introns I and II, including ~2 kb upstream of the native transcriptional start site. For DEX-dependent expression, the Prf open reading frame was digested with Xhol and AvrII and subcloned into the pTA7002 vector (Aoyama and Chua, 1997).

Generation of Prf Fragments

N-term, SD-CC-NBARC-LRR, and CC-NBARC-LRR fragments were amplified using the following combinations of forward and reverse primers, respectively: MT52 (5‘-GGAATTGAGCTCGAGAACAATGGCA-CAAGGAGTGTCGCGAT-3‘) and MT54 (5‘-CTCGAGGAGCTCGAGAACAATGGC-CAAGGAGTGTCGCGAT-3‘) and MT54 (5‘-CTCGAGGAGCTCGAGAACAATGGC-CAAGGAGTGTCGCGAT-3‘) and MT55 (5‘- CTGGAATTCGATCGAGATCGGA-CTG-3‘) and MT51 (5‘-CTCGAGGAGCTCGAGAACAATGGC-CAAGGAGTGTCGCGAT-3‘) and MT50 (5‘-CTCGAGGAGCTCGAGAACAATGGC-CAAGGAGTGTCGCGAT-3‘) and MT51. Open reading frames were then subcloned into...
pTFS40 (Rathjen et al., 1999) under the control of the 3SS promoter and fused to a triple-HA epitope tag.

**Transient Gene Expression and Silencing Assays**

Growth and transient expression conditions for *Nicotiana benthamiana* were as described (Wu et al., 2004) using *Agrobacterium tumefaciens* strain C58C1. For induction of avrPto expression in the transgenic line 291-2 containing Dex:avrPto (Chang et al., 2002), leaves were painted with 30 μM DEX (Sigma-Aldrich) in aqueous solution supplemented with 0.1% (v/v) Tween 20 (Sigma-Aldrich). For VIGS, the procedures of Ratcliff et al. (2001) were followed exactly.

**Protein Analysis**

For the analysis of protein accumulation in planta, leaf samples were ground in liquid nitrogen and solubilized in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA) containing 2 mM DTT and 1% polyvinylpyrrolidone supplemented with a plant protease inhibitor cocktail (Sigma-Aldrich). For detection of AvrPto, 1% (v/v) Triton X-100 was added to the extraction buffer. To raise polyclonal anti-Prf antiserum, a recombinant peptide corresponding to amino acids 761 to 819 was expressed as a fusion with glutathione-S-transferase in Escherichia coli, affinity-purified, and used to immunize rabbits for antisera production. Epitope-tagged proteins were detected variously with anti-HA antibodies, clone 3F10 (Roche); anti-c-myc conjugated to horseradish peroxidase, anti-mouse (DAKO) as appropriate and detected using the peroxidase-conjugated secondary antibody (anti-rabbit, anti-rat [Sigma-Aldrich], or anti-mouse [DAKO]) as appropriate and detected using the enhanced chemiluminescence reagent (GE Healthcare).

**Cloning and Silencing of *N. benthamiana* pto Homologs**

To amplify *Pto*-like sequences from the *N. benthamiana* genome, we synthesized two oligonucleotide primers corresponding to invariant nucleotide sequences in tomato *Pto* homologs (Chang et al., 2002). The forward primer was MS1 corresponding to *Pto* nucleotides 504 to 528, and the reverse primer was MS2 complementary to nucleotides 787 to 813. These primers were used in a PCR (Invitrogen) on *N. benthamiana* genomic DNA to amplify three sequences designated Nb Prf1 to Pth3.

**SEC**

Leaf protein extracts were prepared in extraction buffer as described above containing 5% (v/v) glycerol, 1% polyvinylpyrrolidone, 2 mM DTT, and 1% (v/v) plant protease inhibitor cocktail (Sigma-Aldrich) and cleared by centrifugation at 10,000g followed by filtration through 0.2-μm filters. Crude leaf protein extract (0.5 mL) was injected onto a HiPrep 16/60 Sephacryl S-300 column (GE Healthcare) that had been equilibrated with extraction buffer containing 2 mM DTT at 4°C. The column was calibrated with Bio-Rad gel filtration standards. The column was equilibrated with extraction buffer containing 2 mM DTT at 4°C. The column was calibrated with Bio-Rad gel filtration standards (No. 151-1901), and the void volume was estimated by the elution profile of Blue Dextran (2000K). For protein gel blots, 250 μL of each fraction was precipitated with 1250 μL of cold acetone and resuspended in 1× SDS loading buffer. Immunodetection was performed as described above, using the Femto reagent (Pierce).

**Generation of Transgenic Tomato Lines**

Tomato cv MoneyMaker plants were transformed as described (McCormick et al., 1986) with a binary vector containing Pro*Prf*:Pto-FLAG (de Vries et al., 2006) or the empty vector. Transformants were selected for kanamycin resistance and grown for the collection of genomic DNA and T2 seeds. Homozygous single-insertion transformants were selected in the T3 generation.

**Coimmunoprecipitation Assays**

Protein extracts from *N. benthamiana* or tomato leaves were prepared as described above except that 10% glycerol was added to the extraction buffer. Extracts were centrifuged at 15,000g for 10 min at 4°C. Supernatants were subjected to ultracentrifugation at 100,000g for 20 min at 4°C to remove particulate material or passed through a 0.45-μm filter. Extracts were incubated with antibody-conjugated beads as indicated in the figure legends. Before use, affinity matrices were blocked with 1% BSA. Extracts were mixed with affinity matrices for 2 h at 4°C, with constant rotation to ensure mixing. Affinity matrices were washed four times with 1 mL of extraction buffer containing 250 mM NaCl. Proteins were stripped from the bead fraction by boiling in SDS loading buffer. Elution from anti-FLAG beads was performed by incubating an anti-FLAG M2 affinity gel with equilibration buffer containing 10% glycerol and 200 μg/mL FLAG peptide (Sigma-Aldrich) for 10 min at 25°C with agitation.

**Dephosphorylation Assays**

Dephosphorylation assays were performed at 30°C for 10 min in a buffer containing 300 μg/mL BSA, 3 mM DTT, 200 mM NaCl, 8 mM MnCl2, 3 mM EDTA. 0.1 mM EGTA, 0.01% Brij 35, and 5 units/μL λ protein phosphatase (New England Biolabs). Phosphatase inhibitors were added to final concentrations of 50 mM NaF, 10 mM NaVO4 (sodium orthovanadate), and 50 mM EDTA.

**Trypan Blue Staining**

Fresh tissue was boiled for 2 min in lactophenol (10% [v/v] lactic acid, 10% [v/v] glycerol, 60% [v/v] ethanol, and 10% [v/v] liquid phenol) containing 0.067% (v/v) Trypan blue and cooled to 25°C over 30 min. Stained tissue was cleared by overnight incubation in 2.5 g/mL chloral hydrate and stored in 60% (v/v) glycerol.

**Accession Numbers**

Sequence data from this article can be found in the GenBank data library under the following accession numbers: Prf (U65391), Pto (DQ019170), avrPtoB (U65391), Nb Prf1 (DQ374448), Nb Pth2 (DQ374449), and Nb Pth3 (DQ374450).

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure 1.** ptoGSO2 Does Not Enhance Constitutive Signaling Attributable to 3SS:Prf Expression but Retains Ligand-Dependent Signaling.
- **Supplemental Figure 2.** Disease Assays on Transgenic Lines Generated in This Study.
- **Supplemental Figure 3.** Detection of Prf in Plant.

**ACKNOWLEDGMENTS**

We thank Adriana Bernal (University of California, Davis) for the kind gift of the avrPtoB construct, Majia Sierla for cloning and sequencing Nb Pth1 to Pth2, Esperanza Torres and Chantal Binda for establishing the SEC-FPLC system, and Dagmar Hann for generating the anti-Prf.
antibody. The support of the Gatsby Charitable Foundation is gratefully acknowledged.

Received May 11, 2006; revised July 21, 2006; accepted September 15, 2006; published October 6, 2006.

REFERENCES


The Tomato NBARC-LRR Protein Prf Interacts with Pto Kinase in Vivo to Regulate Specific Plant Immunity
Tatiana S. Mucyn, Alfonso Clemente, Vasilios M.E. Andriotis, Alexi L. Balmuth, Giles E.D. Oldroyd, Brian J. Staskawicz and John P. Rathjen

*Plant Cell* 2006;18;2792-2806; originally published online October 6, 2006;
DOI 10.1105/tpc.106.044016

This information is current as of July 12, 2017

| Supplemental Data | /content/suppl/2006/09/27/tpc.106.044016.DC1.html |
| References | This article cites 47 articles, 17 of which can be accessed free at: /content/18/10/2792.full.html#ref-list-1 |
| eTOCs | Sign up for eTOCs at: http://www.plantcell.org/cgi/alerts/ctmain |
| CiteTrack Alerts | Sign up for CiteTrack Alerts at: http://www.plantcell.org/cgi/alerts/ctmain |
| Subscription Information | Subscription Information for *The Plant Cell* and *Plant Physiology* is available at: http://www.aspbf.org/publications/subscriptions.cfm |