

# Proteolysis of a Negative Regulator of Innate Immunity Is Dependent on Resistance Genes in Tomato and *Nicotiana benthamiana* and Induced by Multiple Bacterial Effectors

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**RPM1-interacting protein 4 (RIN4), a negative regulator of the basal defense response in plants, is targeted by multiple bacterial virulence effectors. We show that RIN4 degradation is induced by the effector AvrPto from *Pseudomonas syringae* and that this degradation in Solanaceous plants is dependent on the resistance protein, Pto, a protein kinase, and Prf, a nucleotide binding site–leucine-rich repeat protein. Our data demonstrate overlap between two of the best-characterized pathways for recognition of pathogen virulence effectors in plants. RIN4 interacts with multiple plant signaling components and bacterial effectors in yeast and in planta. AvrPto induces an endogenous proteolytic activity in both tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* that degrades RIN4 and requires the consensus site cleaved by the protease effector AvrRpt2. The interaction between AvrPto and Pto, but not the kinase activity of Pto, is required for proteolysis of RIN4. Analysis of many of the effectors comprising the secretome of *P. syringae* pv *tomato* DC3000 led to the identification of two additional sequence-unrelated effectors that can also induce degradation of RIN4. Therefore, multiple bacterial effectors besides AvrRpt2 elicit proteolysis of RIN4 in planta.**

## INTRODUCTION

Both plants and animals have defense systems comprised of basal defense responses triggered by detection of common components of potential pathogens (pathogen-associated molecular pattern [PAMP]-triggered immunity) as well as more specific defenses induced by recognition of ligands produced by individual pathogens (effector-triggered immunity) (Jones and Dangl, 2006; McDowell and Simon, 2008). While in vertebrates the adaptive immune system has evolved to supplement innate immunity, plants have elaborated their resistance machinery to provide defense responses that are qualitatively and quantitatively more extreme than the PAMP-elicited resistance (Jones and Takemoto, 2004; Jones and Dangl, 2006). In animals, Toll-like receptors and nucleotide binding oligomerization domain (NOD) proteins extracellularly and intracellularly recognize conserved ligands shared by many pathogens and trigger innate immunity (Athman and Philpott, 2004). There are ~25 NOD-encoding genes in mammals, and their predominant role seems to be in innate immunity (Inohara and Nunez, 2003). In plants, FLAGELLIN SENSITIVE2, a Toll-like receptor-like protein, recognizes flagellin from multiple bacterial pathogens and triggers PAMP-elicited resistance (Gomez-Gomez et al., 2001; Göhre and Robatzek, 2008). The number of genes encoding a nucle-


otide binding site (NBS) domain, the equivalent of the animal NOD domain, is much greater in plants than animals; however, NBS-encoding genes have so far been shown to be involved in recognition of specific pathogen ligands rather than in PAMP-elicited resistance (Meyers et al., 2003).


To be successful, pathogens must suppress the induction of PAMP-elicited resistance. This has been best characterized for pathogenic Gram-negative bacteria, some of which have been shown to secrete up to 40 effectors into the cytosol of their plant or animal hosts (Chang et al., 2004; Lindeberg et al., 2006; Schechter et al., 2006; Vinatzer et al., 2006). Some of these effectors target components of the defense signaling pathways, and plants have evolved to detect perturbations in these pathways (Mudgett, 2005; Grant et al., 2006; Zhou and Chai, 2008). Specific recognition of a bacterial effector elicits a series of plant defense responses often including callose deposition, the release of active oxygen species, induction of defense gene expression, and the hypersensitive response (HR). These defense responses limit pathogen invasion of the plant (Dangl and Jones, 1998).

In one of the best-characterized plant–pathogen interactions, the effectors AvrRpm1 from *Pseudomonas syringae* pv *maculicola* and AvrB from *P. syringae* pv *glycinea* cause the phosphorylation of RPM1-interacting protein 4 (RIN4), a negative regulator of innate immunity in *Arabidopsis thaliana* (Mackey et al., 2002; Kim et al., 2005b), which is then detected by the NBS-leucine-rich repeat (LRR) protein RPM1; this recognition in turn leads to the HR. Elicitation of the HR by RPM1 is abrogated by the proteolytic cleavage of RIN4 by another bacterial effector, AvrRpt2, from *P. syringae* pv *tomato*; however, the disappearance of RIN4 can be detected by a second NBS-LRR protein, RPS2, and, thereby, elicit the HR (Axtell et al., 2003; Mackey

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et al., 2003). Another well-characterized plant-pathogen interaction involves the recognition of the effectors AvrPto and AvrPtoB from *P. syringae* pv *tomato* by Pto in tomato (*Solanum lycopersicum*; Scofield et al., 1996; Tang et al., 1996). Pto is a protein kinase that binds to AvrPto and AvrPtoB and requires Prf, an NBS-LRR protein, to elicit the HR in tomato (Scofield et al., 1996; Tang et al., 1996; Kim et al., 2002). Pto has also been shown to interact with Prf in a multimeric complex in planta and inactivate the E3 ligase activity of AvrPtoB by phosphorylation (Mucyn et al., 2006; Ntoukakis et al., 2009). AvrPto has not been reported to elicit the HR in *Arabidopsis*; however, transgenic expression of AvrPto in *Arabidopsis* suppresses basal defense responses (Hauck et al., 2003; Xing et al., 2007; Gohre et al., 2008; Shan et al., 2008; Xiang et al., 2008).

The effect of AvrPto on basal defense responses in *Arabidopsis* as well as our results from a yeast two-hybrid screen between plant signaling components and bacterial effectors (L. Williams, K. Cavanaugh, J. Greenberg, and R.W. Michelmore, unpublished data) led us to investigate the possible involvement of RIN4 in the Pto-mediated resistance response pathway. In this article, we describe the identification of interactions between RIN4 from both *Arabidopsis* (At RIN4) and a RIN4 homolog from tomato (SI RIN4) and unrelated bacterial effectors, including AvrPto and AvrPtoB, using the yeast two-hybrid system. These interactions were recapitulated in planta using coimmunoprecipitation. Furthermore, in planta studies demonstrated that RIN4 degradation was elicited by AvrPto in a Pto- and Prf-dependent manner. Degradation of RIN4 occurred as the result of endogenous proteolytic activity and involved a similar site as the one involved in the cleavage that occurs in response to the bacterial effector AvrRpt2. Multiple experiments demonstrated that the AvrPto-mediated degradation of RIN4 was not a consequence of general proteolysis associated with an HR. Decreased expression of SI RIN4 in tomato resulted in enhanced resistance to *P. syringae* pv *tomato* expressing *avrPto*. The nonpathogenic wild-type *Pseudomonas fluorescens* did not cause the disappearance of RIN4 in *Nicotiana benthamiana*; ectopic expression and secretion of

AvrPto by *P. fluorescens* resulted in proteolysis of RIN4. The disappearance of RIN4 caused by *P. syringae* pv *tomato* in tomato is dependent on the type III secretion system (T3SS). Screening of the secretome of *P. syringae* pv *tomato* DC3000 identified two additional effectors that elicit degradation of RIN4 in *N. benthamiana*. Therefore, RIN4 is a common and important host target for pathogen effectors and plays a potential role in AvrPto-Pto-mediated resistance in tomato.

## RESULTS

### Cloning of SI RIN4 and Its Enhanced Expression in Plants Challenged by *P. syringae* pv *syringae* B728A

A full-length homolog of RIN4 (SI RIN4) was amplified from tomato cv Rio-Grande 76R (RG-76R) using oligonucleotide primers based on the sequence TC174419 in The Institute for Genomic Research database, cloned, and sequenced. SI RIN4 was 37% identical to the *Arabidopsis* RIN4 (At RIN4) at the amino acid level and was the reciprocal best hit to At RIN4 in BLASTP analyses (see Supplemental Figure 1 online). SI RIN4 contains the cleavage sites for AvrRpt2 at the N and near the C termini (Chisholm et al., 2005).

SI RIN4 is expressed at a moderate level in unchallenged tomato RG-76R, similar to the housekeeping gene encoding  $\alpha$ -tubulin (see Supplemental Figure 2 online). When tomato plants were challenged with the virulent pathogen, *P. syringae* pv *tomato* T1, the transcription level of SI RIN4 was enhanced at least four times, as determined by quantitative real-time RT-PCR (see Supplemental Figure 3 online). Challenge with the avirulent pathogen, *P. syringae* pv *syringae* B728A, enhanced the transcription level of SI RIN4 10 times more than challenge with *P. syringae* pv *tomato* T1 (see Supplemental Figure 3 online). Microarray data described in The Arabidopsis Information Resource ([http://www.Arabidopsis.org/servlets/TairObject?id=1007966202&dtype=expression\\_set](http://www.Arabidopsis.org/servlets/TairObject?id=1007966202&dtype=expression_set)) demonstrated that the

**Table 1.** Interacting Partners of RIN4 Orthologs in Yeast<sup>a</sup> and in Planta<sup>b</sup>

		Yeast Two-Hybrid Assay							Coimmunoprecipitation		
		Bait <sup>c</sup>							<i>N. benthamiana</i>		
		AvrB	AvrPto	AvrPtoB	AvrPtoB (deltaC)	AvrRpm1	RPM1 (N-terminus)	Pto	3FLAG: SIRIN4	3HA: SIRIN4	
Prey	SI RIN4	SI	WI	I	I	WI	SI	WI	AvrPto:3HA	I	
	At RIN4	SI	N	N	WI	N	SI	N	AvrPtoB(deltaC)	I	
	EV	N	N	N	N	N	N	N	Pto	WI	

<sup>a</sup>For the yeast two-hybrid assay, interactions were tested using SI RIN4, At RIN4 in pSLR4, and AvrPto and the other proteins expressed using the bait vector pSLR3 following standard protocols for the LexA system. Empty vector controls and numerous other resistance signaling components, such as NDR1, NPR1, and multiple bacteria effectors, such as HopPsyE, showed no interactions with either At RIN4 or SI RIN4 (data not shown).

<sup>b</sup>For coimmunoprecipitations, *A. tumefaciens* carrying constructs to express 3HA:AvrPtoB(deltaC), AvrPto:3HA, or empty vector (EV) and 3FLAG: SIRIN4 or 3HA:SIRIN4 and Pto were coinfiltrated into *N. benthamiana*. Protein complexes were immunoprecipitated using anti-HA antibody matrix and immunoblotted using either anti-HA or anti-FLAG antibodies. Empty vector was used as a negative control; those data were not included in this table.

<sup>c</sup>Explanations of bait construct abbreviations: AvrPtoB(deltaC), the N terminus only (amino acids 1 to 308) of AvrPtoB; N-RPM1(N-terminus), the N-terminal 176-amino acid region of RPM1. The C-terminus of AvrPtoB (amino acids 308 to 543) was unstable in yeast and therefore could not be tested using the yeast two-hybrid assay. SI, strong interaction; WI, weak interaction; I, interaction; N, no interaction detected.

**Table 2.** Pto-Dependent Degradation of RIN4 in *N. benthamiana*

		<i>N. benthamiana</i>			
		Wild Type		35S:Pto <sup>a</sup>	
		3HA:AtRIN4	3HA:SIRIN4	3HA:AtRIN4	3HA:SIRIN4
AvrPto	P <sup>b</sup>	P		D <sup>c</sup>	D
AvrPtoB	P	P		P	P
Pto <sub>L205D</sub>	D	D		–	– <sup>d</sup>

<sup>a</sup>*N. benthamiana* plants constitutively expressing *Pto* from tomato.

<sup>b</sup>The presence of 3HA:SIRIN4 as detected by immunoblotting.

<sup>c</sup>Degradation of 3HA:SIRIN4.

<sup>d</sup>No experiment was done with this combination.

transcription level of *At RIN4* was similarly enhanced after challenge of *Arabidopsis* with *P. syringae* pv *phaseolicola* and the *P. syringae* pv *tomato* DC3000 *hrcC* mutant. The *hrcC* mutant contains a nonfunctional T3SS and is therefore nonpathogenic (Collmer et al., 2000).

### RIN4 Interacts with Multiple Plant Signaling Components and Bacterial Effectors in Yeast and in Planta

As part of a large yeast two-hybrid screen to investigate interactions between resistance proteins, signaling components, and effector proteins, we identified several interactions between *At RIN4*, *SI RIN4*, plant signaling proteins, and bacterial effectors (Table 1; see Supplemental Figure 4A online). Both *At RIN4* and *SI RIN4* interacted with *AvrB* and the N-terminal 176-amino acid region of *RPM1*. A weak interaction was detected between *SI RIN4* and *Pto*. *SI RIN4* but not *At RIN4* interacted with *AvrPto* and full-length *AvrPtoB*. Both *At RIN4* and *SI RIN4* interacted strongly with the N-terminal region of *AvrPtoB* [*AvrPtoB*(deltaC), amino acids 1 to 308; Table 1]. This suggested that *SI RIN4* is a potential target of *AvrPto* and *AvrPtoB*.

Coimmunoprecipitation confirmed the yeast two-hybrid results. To test the association of *SI RIN4* with the N-terminal region of *AvrPtoB* or *AvrPto*, *SI RIN4* with three FLAG tags at its N terminus (3FLAG:SIRIN4) was transiently coexpressed in *N. benthamiana* with the N-terminal region of *AvrPtoB* [*AvrPtoB*

(deltaC)] or *AvrPto* with 3x hemagglutinin (HA) tags at their N [3HA:AvrPtoB(deltaC)] and C (AvrPto:3HA) termini, respectively, using *Agrobacterium tumefaciens*-mediated transient assays. 3FLAG:SIRIN4 was detected in the pellet following immunoprecipitation of 3HA:AvrPtoB(deltaC) or *AvrPto*:3HA but not in the pellet of the empty vector control (Table 1; see Supplemental Figures 4B and 4C online). To test the association between *SI RIN4* and *Pto*, 3HA:SIRIN4 was transiently expressed in a stable transgenic of *N. benthamiana* overexpressing *Pto*. *Pto* was detected in the pellet following immunoprecipitation of 3HA:SIRIN4 but not in the control pellet (Table 1; see Supplemental Figure 4D online). These data indicated that *SI RIN4* can associate with *Pto*, *AvrPto*, and the N terminus of *AvrPtoB* in planta.

### RIN4 Modification Is Induced in Planta by Three Bacterial Effectors: *AvrRpt2*, *AvrPto*, and *AvrPtoB*

To explore the biological significance of the protein–protein interactions observed in yeast and plants, we studied the effect of four bacterial effectors on the modification of *RIN4* in planta. Both the *SI RIN4* and *At RIN4* proteins were degraded in *N. benthamiana* when coexpressed with *AvrRpt2* using *Agrobacterium*-mediated transient expression (see Supplemental Figure 5 online) as expected due to the conservation of the protease cleavage sites between *SI RIN4* and *At RIN4*. *AvrPto* also induced the degradation of *At RIN4* and *SI RIN4*; however, unlike the situation with *AvrRpt2* (Axtell et al., 2003; Mackey et al., 2003; Chisholm et al., 2005; Kim et al., 2005a), we found that proteolytic degradation associated with *AvrPto* was dependent on the presence of *Pto* and *Prf* (Tables 2 and 3, Figure 1A). *RIN4* degradation was induced by *AvrPto* in transgenic *N. benthamiana* expressing *Pto* but not in wild-type plants (Table 2; see Supplemental Figure 6A online), which contain a *Prf* capable of signaling *AvrPto* recognition (Salmeron et al., 1996). Similarly, *AvrPto* elicited the degradation of *RIN4* in wild-type tomato cv *RG-76R* but not the isogenic mutants, *RG-pto11* and *RG-prf3*, that have inactivating mutations in *Pto* and *Prf*, respectively (Table 3, Figure 1). In addition, the constitutive gain-of-function mutant *Pto*<sub>L205D</sub>, which mimics the negative charge conferred by autophosphorylation of *Pto* and elicits an *AvrPto*-independent, *Prf*-dependent HR (Rathjen et al., 1999), also induced

**Table 3.** Pto- and Prf-Dependent Degradation of RIN4 in Tomato

	Tomato					
	RG-76R		RG-pto11 <sup>a</sup>		RG-prf3 <sup>b</sup>	
	3HA:AtRIN4	3HA:SIRIN4	3HA:AtRIN4	3HA:SIRIN4	3HA:AtRIN4	3HA:SIRIN4
AvrPto	D <sup>c</sup>	D	P <sup>d</sup>	P	P	P
AvrPtoB	D	D	P	P	P	P
Pto <sub>L205D</sub>	– <sup>e</sup>	D	–	–	–	P

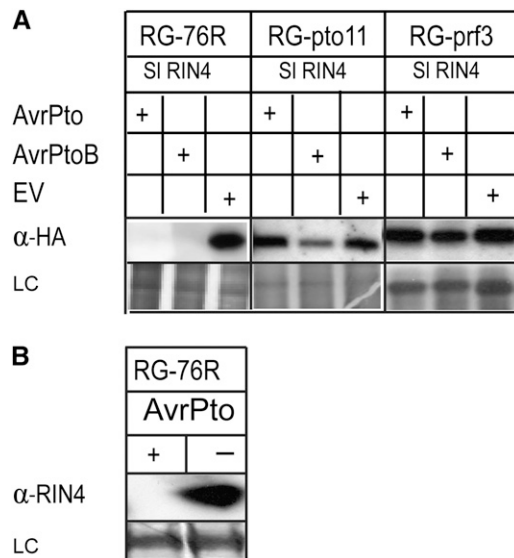
<sup>a</sup>Isogenic line of *RG-76R* with a null allele of *Pto*.

<sup>b</sup>Isogenic line of *RG-76R* with a null allele of *Prf*.

<sup>c</sup>Degradation of 3HA:SIRIN4 or 3HA:AtRIN4 as detected by immunoblotting.

<sup>d</sup>The presence of 3HA:SIRIN4 or 3HA:AtRIN4 as detected by immunoblotting.

<sup>e</sup>No experiment was done with this combination.



**Figure 1.** AvrPto Caused the Proteolysis of SI RIN4 in Tomato.

**(A)** AvrPto- and AvrPtoB-triggered degradation of RIN4 was dependent on Pto and Prf in tomato. 3HA:SI RIN4 and AvrPto, AvrPtoB, or empty vector (EV) were transiently expressed using *A. tumefaciens* in RG-76R tomato plants and RG-76R plants with mutations in *Pto* (RG-pto11) or *Prf* (RG-prf3). Twenty-four hours after infiltration, samples were collected and immunoblotted using anti-HA ( $\alpha$ -HA) antibody to detect the presence of SI RIN4. LC, silver staining of the membrane to show equal loading.

**(B)** AvrPto caused the proteolysis of SI RIN4 expressed from its native promoter in tomato. AvrPto (+) or empty vector (-) were transiently expressed by *A. tumefaciens* in RG-76R tomato plants. Twenty-four hours after infiltration, samples were collected and immunoblotted using anti-At RIN4 ( $\alpha$ -RIN4) antibody to detect native SI RIN4. LC, Ponceau S staining of the membrane to show equal loading.

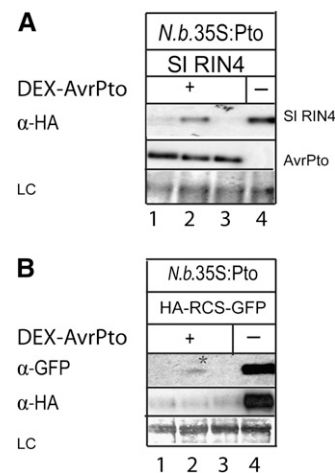
degradation of SI RIN4 in tomato RG-76R but not RG-prf3 (Table 3; see Supplemental Figure 6B online). Therefore, Pto/Prf-dependent degradation of RIN4 appeared to be due to endogenous proteolytic activity rather than a protease activity of AvrPto.

To demonstrate that AvrPto induced the degradation of endogenous SI RIN4 expressed from SI *RIN4* using its native promoter in tomato (and to exclude the possibility that the degradation of SI RIN4 was an artifact of transient overexpression of SI *RIN4* from the cauliflower mosaic virus [CaMV] 35S promoter), we transiently expressed AvrPto in RG-76R tomato plants. Samples were immunoblotted using At RIN4 antibody to detect endogenous levels of SI RIN4. SI RIN4 had been degraded in leaves expressing AvrPto but was present in samples from leaves infiltrated with the empty vector control (Figure 1B).

#### RIN4 Degradation Elicited by AvrPto Involves the Proteasomal Pathway

To determine whether the lack of detection of SI RIN4 was due to the removal of the HA tag or other events, we tested the effect of two inhibitors on the disappearance of SI RIN4. The general protease inhibitor cocktail developed for inhibition of plant pro-

teases did not block the disappearance of SI RIN4 elicited by AvrPto (Figure 2A). However, MG132, a proteasomal inhibitor, did prevent the disappearance of SI RIN4 (Figure 2A). The expression level of AvrPto did not differ between treatments. The experiment was repeated using an artificial substrate with the RIN4 cleavage site (RCS) between the HA tag and green fluorescent protein (GFP; see below). In this case, MG132 did not block the cleavage on the RCS of the artificial substrate (Figure 2B); however, in multiple experiments, the proteolysis of GFP was not complete, possibly indicative of partial inhibition of degradation by MG132. Therefore, the degradation of SI RIN4 induced by AvrPto seems to involve both a protease activity at the AvrPto cleavage site that is not sensitive to the general protease inhibitor cocktail and the proteasomal degradation pathway. However, the role of the proteasomal pathway does not seem to be simply degradation of the product following protease cleavage. Neither MG132 nor the protease inhibitor cocktail blocked the HR caused by AvrPto (data not shown).

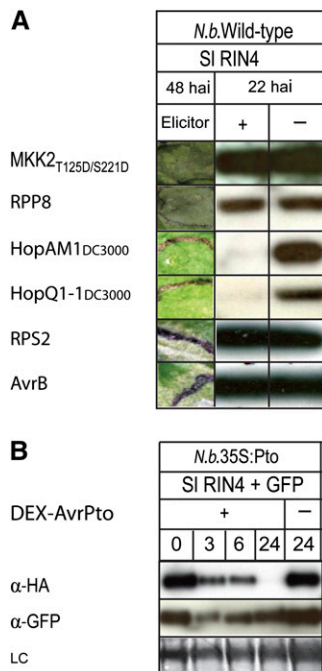


**Figure 2.** Degradation of RIN4 Elicited by AvrPto Involves the Proteasomal Pathway.

In both experiments, 1 d after infiltration, leaves were infused with protease inhibitor (lane 1), MG132 proteasomal inhibitor (lane 2), or DMSO (lanes 3 and 4); DEX was applied to induce expression of *avrPto* 2 h later. Tissues were harvested 4 h after induction with DEX, and immunoblotting was performed using anti-HA antibody ( $\alpha$ -HA) or anti-GFP antibody ( $\alpha$ -GFP). LC, Ponceau S staining of the membrane to show equal loading.

**(A)** The proteasomal inhibitor MG132 partially blocked the degradation of RIN4 elicited by AvrPto. *A. tumefaciens* carrying a gene encoding HA-tagged SI RIN4 was simultaneously coinfiltrated with either *A. tumefaciens* carrying HA-tagged *avrPto* expressed from a DEX-inducible promoter (+) or with *A. tumefaciens* carrying the empty vector (-) into *N. benthamiana* expressing Pto.

**(B)** MG132 partially blocked the proteolysis of GFP after its being cleaved at the RCS. *A. tumefaciens* carrying a gene to express an artificial substrate (see Figure 5A) was simultaneously coinfiltrated either with *A. tumefaciens* carrying *avrPto* expressed from a DEX-inducible promoter (+) or with *A. tumefaciens* carrying the empty vector (-) into *N. benthamiana* expressing Pto. A weak band of GFP was detected as marked by an asterisk.



**Figure 3.** RIN4 Degradation Is Not Due to General Proteolysis.

**(A)** The proteolysis of SI RIN4 is not correlated with the HR phenotype. MKK2<sub>T125D/S221D</sub>, RPP8, HopAM1<sub>DC3000</sub>, HopQ1-1<sub>DC3000</sub>, RPS2, AvrB (+) or empty vector (–), and HA-tagged SI RIN4 were transiently coexpressed in wild-type *N. benthamiana*. The leaf tissues were collected 22 h after infiltration (hai), and immunoblotting was performed using anti-HA antibody. The macroscopic necrosis (HR) phenotype was photographed 48 h after infiltration.

**(B)** Proteolysis of SI RIN4 elicited by AvrPto was not the result of general proteolysis due to a HR. HA-tagged SI RIN4 and GFP were coexpressed with AvrPto under a DEX-inducible promoter (DEX-AvrPto) in transgenic *N. benthamiana* leaves that overexpressed Pto (*N.b.* 35S:Pto). After 24 h, expression of *avrPto* was induced by applying DEX to the leaves. Plant proteins were extracted at 0, 3, 6, and 24 h after DEX induction and assayed by immunoblotting with anti-HA (α-HA) or anti-GFP (α-GFP) antibodies. No SI RIN4 was detected 24 h after the application of DEX, but GFP was readily detected.

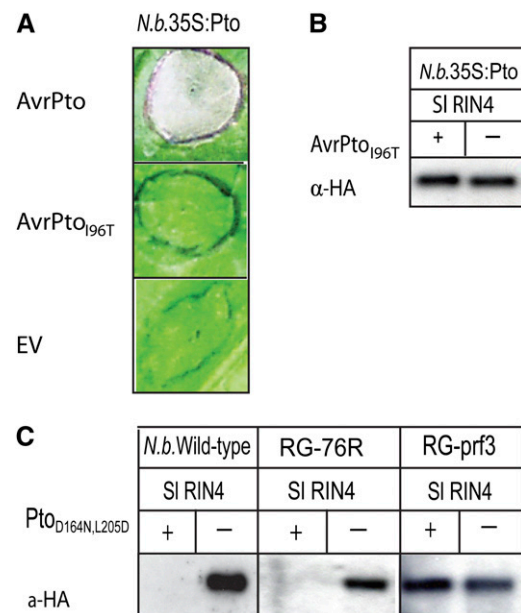
Consequently, the degradation of SI RIN4 appears not to be required for the elicitation of the HR.

### RIN4 Degradation Is Not Due to General Proteolysis Occurring as Part of the HR

To exclude the possibility that AvrPto-mediated degradation of RIN4 was a secondary consequence of the induction of HR or a component of HR induced by multiple specific resistance reactions, we analyzed degradation of SI RIN4 in the presence of several elicitors of HR in *N. benthamiana*. These elicitors of HR included the products of the bacterial avirulence gene *avrB* or those of the genes encoding the NBS-LRR proteins, *RPP8* or *RPS2*, and an auto-activated mutant of a mitogen-activated

protein kinase kinase 2 from tomato (MKK2). None of these elicitors of HR caused the degradation of SI RIN4 (Figure 3A). Therefore, the initiation of the HR does not necessarily elicit RIN4 degradation. In addition, we transiently coexpressed AvrPto under a dexamethasone (DEX)-inducible promoter, SI RIN4, and GFP in transgenic *N. benthamiana* plants that constitutively expressed Pto. SI RIN4 was undetectable 24 h after induction of AvrPto expression, whereas GFP showed a strong band at this time point (Figure 3B). Consequently, AvrPto-induced degradation of RIN4 is specific rather than the result of general proteolysis.

Two additional effectors, HopQ1-1<sub>DC3000</sub> (HopPtoQ<sub>DC3000</sub>) and HopAM1<sub>DC3000</sub> (AvrPpiB<sub>DC3000</sub>) (<http://pseudomonas-syringae>).



**Figure 4.** Pto and AvrPto Interaction but Not Pto Kinase Activity Is Required for RIN4 Proteolysis.

**(A)** AvrPto<sub>I96T</sub> cannot trigger HRs in *N. benthamiana* expressing Pto. AvrPto<sub>I96T</sub> has a polar amino acid substitution in the GINP Ω loop. This mutant does not interact with Pto in the yeast two-hybrid assay. *A. tumefaciens* carrying genes encoding AvrPto<sub>I96T</sub>, AvrPto, or empty vector (EV) were infiltrated into the leaves of *N. benthamiana* expressing Pto. Photographs of the necrosis phenotype were taken 3 d after infiltration.

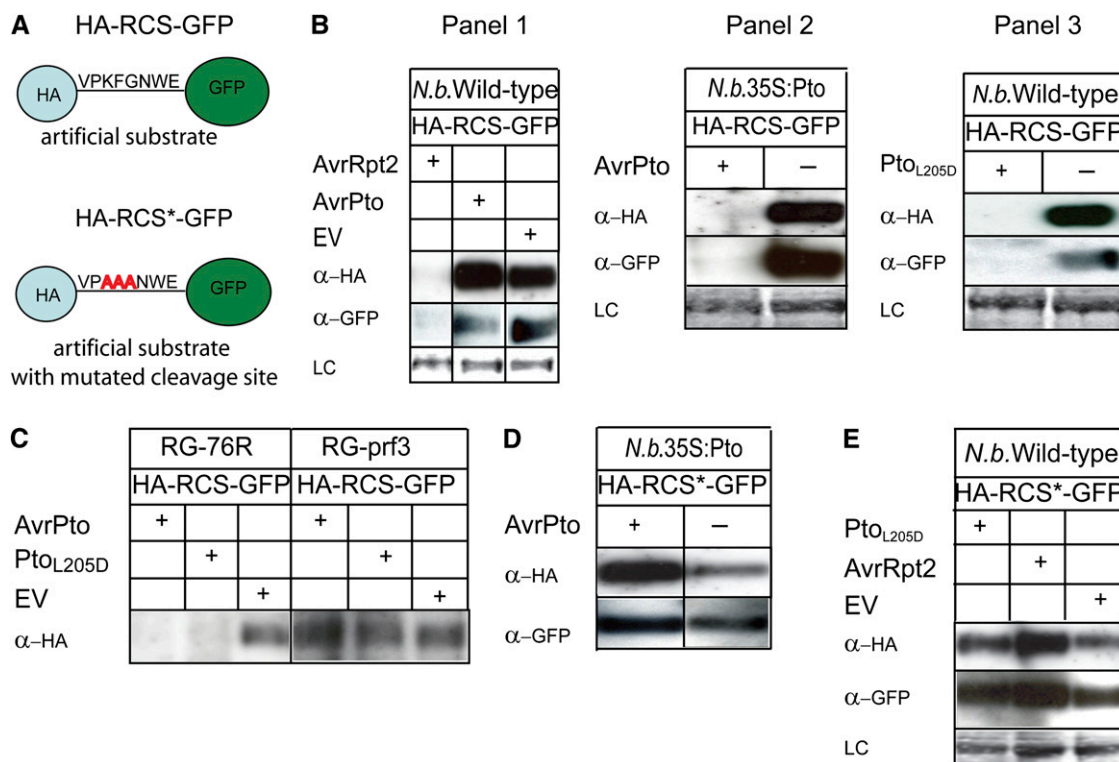
**(B)** Interaction between AvrPto and Pto was required to trigger proteolysis of RIN4 in *N. benthamiana* expressing Pto. HA-tagged SI RIN4 and AvrPto<sub>I96T</sub> (+) or empty vector (–) were transiently coexpressed in transgenic *N. benthamiana* that constitutively overexpressed Pto (*N.b.* 35S:Pto), and immunoblotting was performed using anti-HA antibody (α-HA). AvrPto<sub>I96T</sub> was unable to elicit RIN4 degradation.

**(C)** Kinase activity of Pto is not required for the proteolysis of RIN4. The constitutively active but kinase-deficient mutant Pto<sub>D164N,L205D</sub> and HA-tagged SI RIN4 were transiently coexpressed in wild-type *N. benthamiana*, RG-76R tomato plants, or an isogenic mutant RG-prf3. Total protein was extracted 22 h after coinfiltration, and immunoblotting was performed using anti-HA antibody (α-HA). Pto<sub>D164N,L205D</sub> elicited RIN4 degradation in a Prf-dependent manner.

org/pst\_home.html), that caused the disappearance of RIN4 were identified by screening the secretome of *P. syringae* pv *tomato* DC3000 (see below). Neither effector elicited HR in wild-type *N. benthamiana* 48 h after inoculation (Figure 3A); however, both caused the disappearance of RIN4 (Figure 3A). Therefore, RIN4 degradation occurred independently of HR, confirming that RIN4 degradation was not the result of general proteolysis. HopQ1-1<sub>DC3000</sub> was reported to cause HR 48 h after transient expression (Wei et al., 2007). However, we did not observe HR in multiple inoculations; this may have been due to using different vectors to express HopQ1-1<sub>DC3000</sub>, different experimental conditions, or different genotypes of *N. benthamiana*.

### The Interaction between AvrPto and Pto, but Not the Kinase Activity of Pto, Is Required for Proteolysis of RIN4

To test whether the ability of AvrPto to interact with Pto is required for the proteolysis of RIN4, an AvrPto mutant, AvrPto<sub>196T</sub>, was coexpressed with SI RIN4 in *N. benthamiana*. AvrPto<sub>196T</sub> has a polar amino acid substitution in the GINP  $\Omega$  loop and does not interact with Pto in the yeast two-hybrid assay (Wulf et al., 2004). AvrPto<sub>196T</sub> did not trigger an HR in *N. benthamiana* expressing Pto (Figure 4A). Coexpression of AvrPto<sub>196T</sub> and SI RIN4 in *N. benthamiana* constitutively expressing Pto did not induce the proteolysis of SI RIN4 (Figure 4B). This indicates that interaction between AvrPto and Pto is required for the AvrPto-elicited



**Figure 5.** Proteolysis of an Artificial Substrate Induced in *N. benthamiana* and Tomato.

**(A)** The artificial substrates comprising either the native or mutated RCS between an HA-tag and GFP. Eight amino acids from the first AvrRpt2 cleavage site in RIN4 were placed between an HA-tag and GFP (HA-RCS-GFP). Similarly, a mutated cleavage site with three Ala substitutions at positions known to be important for cleavage by AvrRpt2 was placed between an HA tag and GFP (HA-RCS\*-GFP).

**(B)** AvrPto induced proteolysis of artificial substrates in a Pto-dependent manner in *N. benthamiana*. Panel 1: HA-RCS-GFP was transiently coexpressed with AvrRpt2, AvrPto, or empty vector (EV) in wild-type *N. benthamiana*. Total proteins were extracted before the HR caused by AvrRpt2 or AvrPto was evident, and immunoblotting was performed using anti-HA ( $\alpha$ -HA) or anti-GFP ( $\alpha$ -GFP) antibody. Panel 2: HA-RCS-GFP was transiently coexpressed with AvrPto (+) or empty vector (-) in transgenic *N. benthamiana* expressing Pto. Total proteins were extracted and immunoblotted against anti-HA ( $\alpha$ -HA) antibody or anti-GFP ( $\alpha$ -GFP) antibody. The lack of a detectable HA tag or GFP indicated that the cleavage site in AS is sufficient for AvrPto-induced proteolysis. Panel 3: Constitutively active Pto<sub>L205D</sub> induced proteolysis in *N. benthamiana*. HA-RCS-GFP was coexpressed with Pto<sub>L205D</sub> (+) or empty vector (-) in wild-type *N. benthamiana*, and immunoblotting was performed using anti-HA ( $\alpha$ -HA) or anti-GFP ( $\alpha$ -GFP) antibody.

**(C)** Proteolysis induced by AvrPto or Pto<sub>L205D</sub> is dependent on Prf in tomato. HA-RCS-GFP was coexpressed in RG-76R tomato plants or an isogenic mutant at the *Prf* locus (RG-prf3) with AvrPto (+), Pto<sub>L205D</sub> (+), or empty vector (EV), and immunoblotting was performed using anti-HA ( $\alpha$ -HA) antibody.

**(D)** and **(E)** Amino acid substitutions in the cleavage site prevented cleavage caused by AvrRpt2, AvrPto, or Pto<sub>L205D</sub> in *N. benthamiana*. HA-RCS\*-GFP was coexpressed with AvrRpt2, AvrPto, Pto<sub>L205D</sub>, or empty vector (EV) in wild-type or transgenic *N. benthamiana* expressing Pto, and immunoblotting was performed using anti-HA ( $\alpha$ -HA) or anti-GFP ( $\alpha$ -GFP) antibody. LC, Ponceau S staining of the membrane to show equal loading.

proteolysis of RIN4 in planta or that an unknown modified interaction between AvrPto<sub>196T</sub> and RIN4 abrogates proteolysis.

To determine whether the kinase activity of Pto is required for the degradation of RIN4, we tested a constitutively active, kinase-deficient double mutant, Pto<sub>D164N, L205D</sub>, which does not interact with AvrPto and elicits a strong HR in *N. benthamiana* (Wu et al., 2004). Transient expression of Pto<sub>D164N, L205D</sub> and SI RIN4 in both *N. benthamiana* and tomato RG-76R resulted in the degradation of SI RIN4 (Figure 4C), indicating that the kinase activity of the constitutively active form of Pto is not required for the degradation of RIN4. Coexpression of Pto<sub>D164N, L205D</sub> and SI RIN4 in tomato RG-prf3 did not cause an HR as reported previously (Wu et al., 2004) or proteolysis of SI RIN4 (Figure 4C).

### The Site Cleaved by the Effector AvrRpt2 Is Required for Proteolysis of RIN4

The parallel data from yeast two-hybrid and in planta assays for At RIN4 and SI RIN4, which have very different sequences except at regions containing the AvrRpt2 cleavage sites (see Supplemental Figure 1 online), the dependency of RIN4 degradation on Pto and Prf, and particularly the degradation induced by Pto<sub>L205D</sub> in the absence of AvrPto all indicated that AvrPto does not directly degrade RIN4 (in contrast with the situation with the protease effector AvrRpt2) but rather induces the activation of endogenous proteolytic activity. To test the requirement for the site cleaved by AvrRpt2, we generated an artificial substrate containing the RCS used by AvrRpt2 as well as a control substrate comprising a mutated RCS. Several amino acids in the cleavage site, particularly the Phe at position 4, had previously been shown to be important for AvrRpt2-mediated RIN4 cleavage and subsequent degradation (Chisholm et al., 2005; Kim et al., 2005a; Takemoto and Jones, 2005). For this study, the Lys at position 3, Gly at position 5, and the Phe at position 4 were all changed to Ala. The wild-type RCS or the mutated RCS (RCS\*), both eight amino acids in length, were then inserted between an HA and the GFP to generate HA-RCS-GFP and HA-RCS\*-GFP chimeric proteins, respectively (Figure 5A). When HA-RCS-GFP was coexpressed with AvrPto in wild-type and Pto-expressing *N. benthamiana*, the artificial substrate was detectable in wild-type *N. benthamiana* but not in the transgenic

plants constitutively expressing Pto (Table 4, Figure 5B, Panels 1 and 2). To determine whether the peptides within the AvrRpt2 cleavage site were sufficient to target GFP to the proteasome, we used GFP antibody to assay whether the GFP persisted after cleavage. However, GFP was not detected, indicating that the cleavage site is sufficient to target the fusion protein to the proteasome (Figure 5B, Panel 2). Similarly, transient expression of AvrPto induced the disappearance of HA-RCS-GFP in RG-76R tomato plants but not in RG-prf3 plants (Table 4, Figure 5C). Coexpressing the constitutive mutant Pto<sub>L205D</sub> with HA-RCS-GFP resulted in its disappearance in wild-type *N. benthamiana* and RG-76R tomato, but not in RG-prf3 plants (Table 4, Figures 5B, Panel 3, and 5C). Therefore, the AvrPto-induced disappearance of the artificial substrate required a similar cleavage site as is required for cleavage by AvrRpt2 and was also Pto and Prf dependent. HA-RCS\*-GFP, containing mutations in the cleavage site, was not degraded when coexpressed with AvrPto in *N. benthamiana* expressing Pto (Table 4, Figure 5D). These mutations also blocked the disappearance of HA-RCS\*-GFP when it was coexpressed with Pto<sub>L205D</sub> in wild-type *N. benthamiana* (Table 4, Figure 5E). These data are consistent with the activation of an endogenous proteolytic activity that requires the same cleavage site as does AvrRpt2.

### Decreased Levels of RIN4 Result in Increased AvrPto-Induced Inhibition of Pathogen Growth

We attempted to generate transgenic plants of tomato cv RG-76R with either elevated or reduced levels of RIN4 to determine if RIN4 degradation is required for AvrPto-Pto-initiated resistance. However, we were able to obtain very few transgenic plants despite multiple transformation attempts. Concurrent transformations with other constructs resulted in normal transformation rates. Therefore, it seems that perturbations of RIN4 levels, either increased or decreased, were deleterious in tomato. Only one plant could be obtained that overexpressed RIN4 from the 35S promoter; this plant had severely abnormal morphology, yielded stunted progeny, and could not be studied further. Four independent transgenics were obtained in which the expression of RIN4 had been reduced by ~85% using RNA interference (RNAi). One was sterile and only two of the other three provided

**Table 4.** Pto- and Prf-Dependent Degradation of Artificial Substrates Required an Intact AvrRpt2 Cleavage Site

	<i>N. benthamiana</i>				Tomato	
	Wild Type		35S:Pto <sup>a</sup>		RG-76R	RG-prf3 <sup>b</sup>
	HA-RCS-GFP	HA-RCS*-GFP	HA-RCS-GFP	HA-RCS*-GFP	HA-RCS-GFP	HA-RCS-GFP
AvrPto	P <sup>c</sup>	P	D <sup>d</sup>	P	D	P
AvrRpt2	D	P	– <sup>e</sup>	–	D	P
Pto <sub>L205D</sub>	D	P	–	–	–	–

<sup>a</sup>*N. benthamiana* plants constitutively expressing *Pto* from tomato.

<sup>b</sup>Isogenic line of RG-76R with a null allele of *Prf*.

<sup>c</sup>The presence of artificial substrate detected by immunoblotting.

<sup>d</sup>Degradation of artificial substrate as determined by immunoblotting.

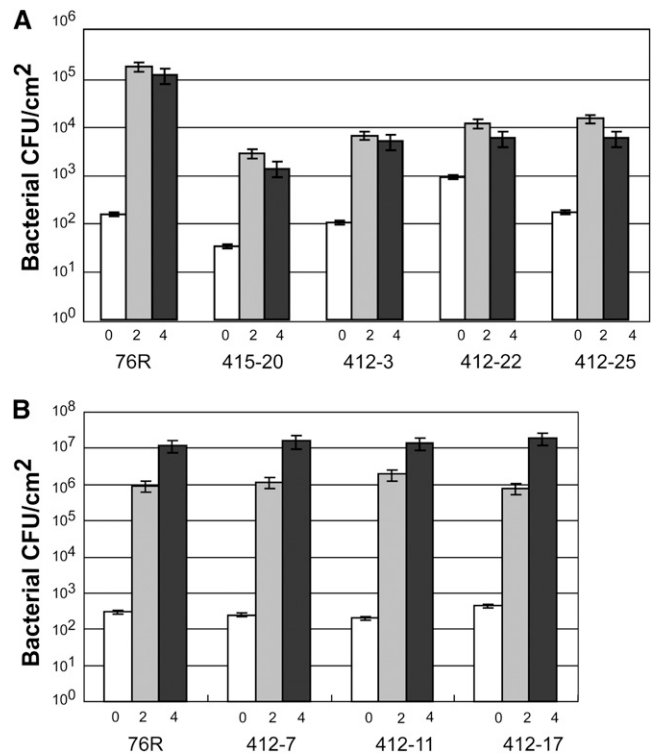
<sup>e</sup>No experiment was done with this combination.

sufficient numbers of T2 seeds for further analysis. T2 plants were derived from T1 plants 412 and 415 that showed a 97 and 85% reduction in expression of RIN4, respectively, as detected by quantitative PCR. Transmission of the T-DNA to the T2 generation was less than Mendelian expectations in both families, further indicating the deleterious effects of abnormal RIN4 levels in tomato; PCR products representing T-DNA containing the complete RNAi construct were detected in only 8 out of 40 T2 plants in the 412-derived family and 5 out of 20 T2 plants in the 415-derived family. The growth of *P. syringae* pv *tomato* T1, expressing AvrPto, was repressed in these silenced T2 plants (Figure 6A). However, the growth of an isogenic *P. syringae* pv *tomato* strain T1 carrying only the empty vector and lacking AvrPto was similar to the wild-type and transgenic plants (Figure 6B). These data are consistent with the decreased expression of SI RIN4 in these transgenic plants specifically enhancing Pto-dependent resistance as opposed to Pto-independent resistance.

#### Additional Effectors Cause Proteolysis of RIN4 in Tomato in a Pto- and Prf-Independent Manner

In an attempt to investigate AvrPto-induced degradation of RIN4 when AvrPto is secreted by *P. syringae* rather than overexpressed in *Agrobacterium*-mediated transient assays, we studied RIN4 degradation using isogenic strains of *P. syringae* pv *tomato* T1, carrying a plasmid with and without *avrPto*. However, *P. syringae* pv *tomato* T1 caused the proteolysis of SI RIN4 in RG-76R, RG-pto11, and RG-prf3 tomato plants regardless of whether *avrPto* was present (Figure 7A). Wild-type *P. syringae* pv *tomato* strain DC3000, expressing AvrPto and AvrPtoB, caused degradation of SI RIN4 in stably transformed *N. benthamiana* plants constitutively expressing SI Pto and induced to express SI RIN4 by applying DEX on the leaves; a mutant strain of DC3000 lacking *avrPto* caused the degradation of SI RIN4 in these transgenic plants as well (Figure 7B, Panel 2). However, *P. syringae* pv *tomato* strain DC3000 *hrcC*, a nonpathogen because it lacks a functional T3SS (Alfano and Collmer, 1997), did not cause the proteolysis of SI RIN4, indicating that the T3SS is required for the proteolysis (Figure 7B, Panel 2). To test whether the lack of accumulation of SI RIN4 in tomato is due to inhibition of agro-transient expression, we inoculated tomato leaves of RG-76R and its isogenic line RG-prf3 with *P. syringae* pv *tomato* strain DC3000, DC3000 lacking *avrPto*, and DC3000 *hrcC*. Both *P. syringae* pv *tomato* strain DC3000 and DC3000 lacking *avrPto* caused the degradation of SI RIN4 but DC3000 *hrcC* did not (Figure 7B, Panel 1). Together, these data suggested that other effectors induced RIN4 degradation independent of Pto and Prf.

To determine which other bacterial effectors induced the degradation of RIN4, we coexpressed SI RIN4 with each of 30 different effectors from *P. syringae* pv *tomato* DC3000 in *N. benthamiana* and assayed for degradation of SI RIN4. We identified two additional effectors, HopQ1-1<sub>DC3000</sub> (HopPto<sub>DC3000</sub>) and HopAM1<sub>DC3000</sub> (AvrPpiB<sub>DC3000</sub>) ([http://pseudomonas-syringae.org/pst\\_home.html](http://pseudomonas-syringae.org/pst_home.html)), that induced the proteolysis of SI RIN4 in wild-type *N. benthamiana* (Figure 3A). Homologs of HopQ1 are present in *P. syringae* pv *tomato* T1



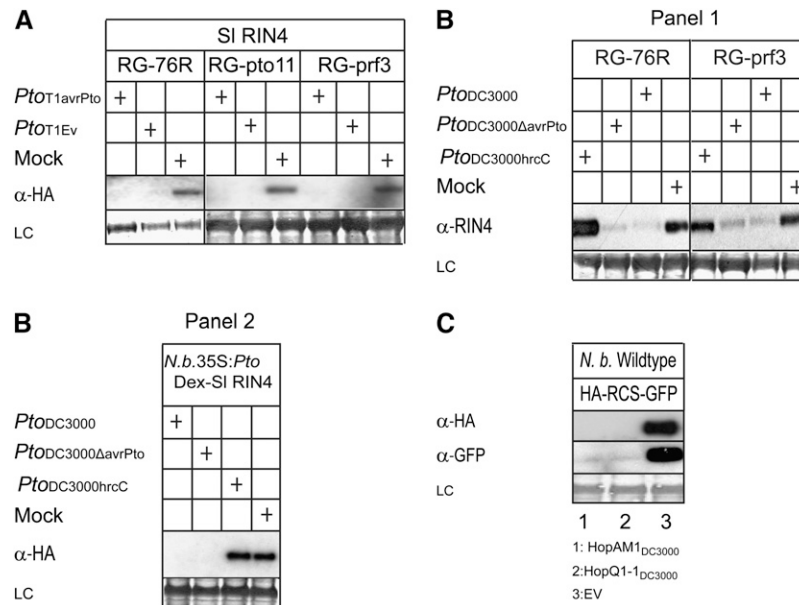
**Figure 6.** Degradation of RIN4 Is Important for AvrPto-Dependent Inhibition of Bacterial Growth.

**(A)** Growth of *P. syringae* pv *tomato* T1 expressing *avrPto* was reduced in RIN4 RNAi-silenced T2 tomato plants. T2 plants derived from original (T1) transgenic plants 415 (one plant) and 412 (three plants) were inoculated by infiltrating the pathogen at  $10^3$  cfu/mL and assayed after 0 (white), 2 (gray), and 4 (black) d; error bars indicate the SD from three biological replications.

**(B)** Wild-type *P. syringae* pv *tomato* T1 exhibited normal levels of growth in RIN4 RNAi-silenced T2 plants. Three transgenic RNAi T2 plants derived from one RNAi-silenced T1 line (412) with reduced RIN4 expression relative to their isogenic wild-type tomato RG-76R were inoculated by infiltrating with pathogen at  $10^3$  cfu/mL and assayed 0, 2, and 4 d after infiltration. Error bars indicate the SD from three measurements.

(<http://staff.vbi.vt.edu/jcslab/pseudomonas/>), and homologs of HopQ1-1 and HopAM1 exist in many strains of *P. syringae* (Lindeberg et al., 2005; Sarkar et al., 2006). Therefore, it is likely that the Pto- and Prf-independent SI RIN4 degradation observed with *P. syringae* pv *tomato* strain T1, as well as that observed with *P. syringae* pv *tomato* strain DC3000, is due to the direct or indirect action of other effectors. Both effectors also induced degradation of the artificial RCS substrate in wild-type *N. benthamiana* (Figure 7C), indicating that the RCS is necessary and sufficient for effectors to elicit RIN4 degradation in a Pto-independent manner. HopAM1<sub>DC3000</sub> caused an HR in tomato but not in *N. benthamiana*, while HopQ1-1<sub>DC3000</sub> did not cause an HR in tomato and elicited chlorosis in *N. benthamiana* (<http://charge.ucdavis.edu/>). These results provide further evidence that the induction of RIN4 degradation by bacterial effectors was not a consequence of the general proteolysis that occurs as part





**Figure 7.** Degradation of RIN4 by *Pseudomonas* spp.

**(A)** *P. syringae* pv *tomato* T1 with or without AvrPto caused proteolysis of SI RIN4 in tomato in a Pto- and Prf-independent manner. *P. syringae* pv *tomato* strain T1 (*Pto*<sub>T1</sub>) with and without *avrPto* (*avrPto* and EV, respectively) were coinoculated with *A. tumefaciens* strain C58C1 with a binary vector that provided expression of HA-tagged SI RIN4 in planta. The negative control (Mock) was infiltrated with 10 mM MgCl<sub>2</sub>. Coinoculations were made with *A. tumefaciens* at OD<sub>600</sub> = 0.5 and *P. syringae* pv *tomato* at OD<sub>600</sub> = 10<sup>-2</sup> into wild-type RG-76R, RG-pto11, and RG-prf3 tomato leaves, and total proteins were extracted 24 h after infiltration and the presence of RIN4 assayed using anti-HA antibody (α-HA). LC, Ponceau S staining of the membrane to show equal loading.

**(B)** *P. syringae* pv *tomato* DC3000 and its derivative lacking AvrPto caused T3SS-dependent proteolysis of RIN4 in tomato and in *N. benthamiana*. Wild-type *P. syringae* pv *tomato* DC3000 (*Pto*<sub>DC3000</sub>), a knockout derivative lacking AvrPto (*Pto*<sub>DC3000</sub>Δ*avrPto*), or a T3SS-deficient mutant (*Pto*<sub>DC3000</sub>hrcC) were individually inoculated at OD<sub>600</sub> = 10<sup>-2</sup> into leaves of tomato RG-76R, RG-prf3, or transgenic *N. benthamiana* constitutively expressing *Pto* and expressing HA-tagged SI RIN4 under the DEX-inducible promoter. In tomato, total protein was extracted and detected using anti-At RIN4 (α-RIN4) antibody (Panel 1). In *N. benthamiana*, 24 h after inoculation, SI RIN4 expression was induced by applying DEX onto the leaves. Total plant protein was extracted 24 h after induction and the presence of RIN4 assayed using anti-HA antibody (α-HA) (Panel 2). LC, Ponceau S staining of the membrane to show equal loading.

**(C)** Effectors HopAM1 and HopQ1-1 from *Pto*<sub>DC3000</sub> cleaved the artificial substrate (HA-RCS-GFP) containing the native RCS independent of Pto. HA-RCS-GFP and HopAM1<sub>DC3000</sub> (lane 1), HopQ1-1<sub>DC3000</sub> (lane 2), or empty vector (lane 3) were coexpressed in wild-type *N. benthamiana* leaves, and immunoblotting was performed using anti-HA antibody (α-HA) or anti-GFP antibody (α-GFP). LC, Ponceau S staining of the membrane to show equal loading.

of the HR. The biochemical, particularly proteolytic, activities of these two effectors are not known and are currently under investigation.

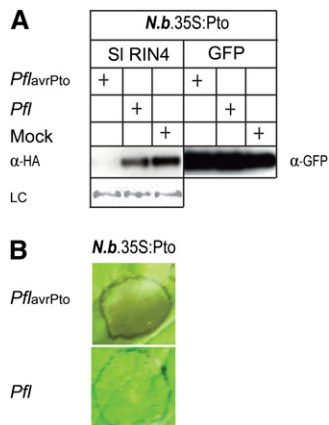
#### RIN4 Is Degraded When AvrPto Is Secreted by *P. fluorescens*

To further investigate AvrPto-induced degradation of RIN4 when secreted rather than overexpressed in *Agrobacterium*-mediated transient assays, we used isogenic strains of transgenic *P. fluorescens*, one carrying a plasmid with and one without *avrPto*. *P. fluorescens* strain 55 is a nonpathogen. It carries pHIR11, which expresses a portion of the pathogenicity island from *P. syringae* pv *syringae* and enables secretion of effectors from *P. fluorescens* (Collmer et al., 2000). Wild-type *P. fluorescens* barely grows in *N. benthamiana*; however, expression of AvrPto at low inoculum concentrations resulted in two- to threefold increased growth of *P. fluorescens* consistent with a virulence phenotype of AvrPto; however, as with *P. syringae* pv *tomato* DC3000 at this

concentration, no degradation of RIN4 was observed (data not shown). On the other hand, inoculation of *P. fluorescens* 55 (pHIR11), expressing or not expressing AvrPto, into *N. benthamiana* at a higher concentration (colony-forming units [cfu] = 10<sup>8</sup>/mL), resulted in the AvrPto-specific degradation of SI RIN4 (Figure 8A). GFP was not degraded in the control inoculations with either strain of *P. fluorescens* (Figure 8A), providing further evidence that RIN4 degradation is specific to the activity of AvrPto rather than a consequence of HR induction. AvrPto-specific induction of HR after 24 h was not observed unless inoculations were made with 10<sup>9</sup> cfu/mL (Figure 8B).

#### DISCUSSION

Proteolysis of RIN4 seems to be an important aspect of plant-bacteria interactions that can be achieved by a variety of mechanisms, including direct enzymatic activity of a bacterial protein



**Figure 8.** AvrPto-Specific Degradation of SI RIN4 by *P. fluorescens* in *N. benthamiana*.

**(A)** AvrPto-specific degradation of SI RIN4 by *P. fluorescens*. Strains of *P. fluorescens* 55 (pHIR11) ( $OD_{600} = 0.1$ ) expressing (*PflavrPto*) or not expressing (*Pfl*) *avrPto* were coinoculated with *A. tumefaciens* carrying SI RIN4 ( $OD_{600} = 1.0$ ) or GFP (as a control) into leaves of transgenic *N. benthamiana* that expressed *Pto*. After 24 h, leaf samples were harvested and immunoblotted using anti-HA ( $\alpha$ -HA) or anti-GFP ( $\alpha$ -GFP) antibodies. The mock treatment (Mock) was infiltration with 10 mM  $MgCl_2$  solution. LC, Ponceau S staining of the membrane to show equal loading. **(B)** *P. fluorescens* at a high inoculum concentration elicited necrosis in an *avrPto*-dependent manner. Strains of *P. fluorescens* 55 (pHIR11) ( $OD_{600} = 1.0$ ) expressing (*PflavrPto*) or not expressing (*Pfl*) *avrPto* were inoculated individually into leaves of transgenic *N. benthamiana* that expressed *Pto*. Only *P. fluorescens* expressing *avrPto* elicited macroscopic necrosis indicative of a HR 48 h after the inoculation.

elicitor or indirectly through induction of the endogenous proteolytic pathway. SI RIN4 seems to be targeted directly or indirectly by multiple effectors from *P. syringae*. At least seven effectors, AvrRpt2, AvrPto, AvrPtoB, HopQ1-1<sub>DC3000</sub> (HopPtoQ<sub>DC3000</sub>), HopAM1<sub>DC3000</sub> (AvrPpiB<sub>DC3000</sub>), AvrB, and AvrRpm1, cause various modifications of SI RIN4. Yeast two-hybrid and coimmunoprecipitation data indicate that AvrPto and RIN4 can directly interact; the importance of this interaction to the elicitation of RIN4 degradation is unclear because degradation can occur in the absence of AvrPto. The experiment with the artificial substrate demonstrated that the site of RIN4 cleavage by AvrRpt2 is required for Pto-AvrPto-mediated degradation and is sufficient to target GFP with the cleavage site at its N terminus to the proteasome (Figure 5B). However, the mechanisms of RIN4 degradation by AvrRpt2 and AvrPto are different. AvrRpt2 is known to function as a protease (Axtell et al., 2003). By contrast, AvrPto is not thought to be a protease, and the constitutive gain-of-function Pto<sub>L205D</sub> mutant caused proteolysis of SI RIN4 in the absence of AvrPto, indicating that proteolysis of RIN4 is not dependent on a proteolytic activity of AvrPto. Also, unlike AvrRpt2-induced cleavage, AvrPto-induced degradation of RIN4 is Pto and Prf dependent, and the interaction between AvrPto and Pto is required for AvrPto-induced cleavage of RIN4.

The role of RIN4 degradation in plant-bacterial interactions is unclear. Multiple lines of evidence demonstrated that the proteolysis of RIN4 elicited by AvrPto is not due to general protein

degradation as part of the HR. Even when there was extensive necrosis 48 h after ectopic expression of several HR inducers, RIN4 degradation was not apparent (Figure 3A). Conversely, RIN4 was degraded well before necrosis in multiple experiments, including when AvrPto was secreted by *P. fluorescens* (Figure 8B). RIN4 has been reported to be a negative regulator of basal resistance in *Arabidopsis* (Kim et al., 2005b). However, our data indicate that the situation is considerably more complex than currently described. Pto and Prf may be part of one or more macromolecular complexes (Mucyn et al., 2006) with multiple negative regulators, including RIN4. One possible role of the Prf-dependent proteolysis of RIN4 is to alleviate basal defense suppression by RIN4 or other related proteins. This scenario is similar to the derepression of WRKY-mediated suppression of basal defenses upon activation of MLA10 (Shen et al., 2007). However, the dynamics, cellular localization, and coincidence of such complexes and their roles in resistance are not currently known. Clearly, AvrPto interacts with RIN4 in the absence of Pto, but this does not trigger RIN4 degradation or resistance. Also, Pto interacts with RIN4 in the absence of AvrPto; however, binding of AvrPto to Pto results a Prf-dependent activation of an endogenous proteolytic pathway that degrades RIN4.

Inhibiting degradation of a negative regulator, such as RIN4, could be anticipated to contribute to virulence. Therefore, the proteolysis of RIN4, directly or indirectly induced by bacterial effectors, is seemingly counterintuitive if this results in increased induction of the resistance response. However, the effector-elicited resistance response is much stronger than the PAMP-induced basal resistance response (Jones and Dangl, 2006). Consistent with AvrPto-elicited RIN4 degradation increasing resistance, downregulation of RIN4 expression in tomato using RNAi suppressed growth of *P. syringae* pv *tomato* specifically expressing AvrPto (Figure 6A). The benefit of proteolysis of RIN4, or of other potential defense regulators that contain the same cleavage site (Chisholm et al., 2005; Takemoto and Jones, 2005), caused directly by AvrRpt2 and indirectly by AvrPto may lie in the abrogation of the detection of RIN4 modification by effectors such as AvrRpm1 and AvrB as exemplified by AvrRpt2 blocking the HR induced by AvrB or AvrRpm1 in the presence of RPM1 in *Arabidopsis* (Ritter and Dangl, 1996). Additional effectors may act by other mechanisms to minimize the effects of removing the negative-regulatory effects of RIN4 on the induction of basal resistance. AvrPto is known to have other Pto- and Prf-independent effects of virulence (Shan et al., 2008; Zipfel and Rathjen, 2008). The effect of AvrPto-mediated RIN4 proteolysis on virulence would only be detected when effectors that modify RIN4 and resistance genes capable of detecting such modifications are present.

Our data provide further evidence for the overlap and interplay of PAMP-triggered and effector-triggered resistance in plants and are consistent with roles for AvrPto and AvrPtoB in modulating PAMP-elicited resistance. Effector-triggered and PAMP-triggered resistance share signaling components in common, including RIN4, and similar sets of response genes are induced (Jones and Dangl, 2006). Effectors that repress PAMP-triggered resistance in one species may elicit an HR in another (Lahaye and Bonas, 2001). RIN4 is both a negative regulator of basal resistance as well as a trigger for HR (Mackey et al., 2003; Kim et al., 2005b). AvrPto and AvrPtoB suppress

PAMP-triggered resistance in compatible interactions but trigger an HR in incompatible ones in a Prf- and Pto-dependent manner (Hauck et al., 2003; He et al., 2006; Hann and Rathjen, 2007). Overexpression of Pto and Prf leads to constitutive, ligand-independent induction of the resistance response or an HR (Salmeron et al., 1996; Tang et al., 1999; Tobias et al., 1999; Xiao et al., 2003). Tomato mutants lacking Prf were more susceptible to virulent strains of *P. syringae* pv *tomato*; one of several explanations of this phenotype is a role for Prf in PAMP-triggered resistance (Chang et al., 2001). It is also possible that Prf is involved in the recognition of other effectors from the virulent strain. This leads to the consideration of what extent Pto/Prf-mediated resistance is similar to or different from other effector-triggered resistance systems and whether Pto and Prf are primarily involved in PAMP- or effector-triggered resistance. In the Pto/Prf pathway, the kinase gene component is multicopy and exhibits intraspecific variation in sequence and function (Riely and Martin, 2001; Rose et al., 2005, 2007). In other specific resistances, the genes encoding the NBS-LRR proteins are the ones that have been duplicated and are the more variable component (McHale et al., 2006). This may reflect the involvement of Pto and Prf in PAMP-triggered resistance that is thought to predate the evolution of specific induction of an HR in plants (Alfano and Collmer, 2004; Jones and Dangl, 2006). The first targets of bacterial effectors were, therefore, likely to have been components of PAMP-elicited resistance. Consequently, RIN4 and Pto homologs may have been early targets for bacterial effectors, and selection on host populations to detect interference with these components could have subsequently resulted in the evolution of specific resistance proteins. The recognition of AvrPto and AvrPtoB and elicitation of HR in tomato is likely, therefore, the more highly evolved situation relative to that in *N. benthamiana*. Consistent with this, coexpression of tomato Prf and Pto in *N. benthamiana* resulted in an HR induced by AvrPtoB (Mucyn et al., 2006).

Several proteins from *Arabidopsis* contain the same proteolytic cleavage site as RIN4 and have been shown to be cleaved by AvrRpt2 (Hirano et al., 1999; Chisholm et al., 2005; Takemoto and Jones, 2005). It will be interesting to determine which and how many of these other proteins are also targeted by the virulence effectors we identified as inducing RIN4 degradation. Some may be functional proteins; others, including RIN4 itself, may be decoys or sentinels for the presence of proteolytic effectors (van der Hoorn and Kamoun, 2008). The presence of multiple proteins targeted by many functionally redundant effectors leads to several possible evolutionary questions and scenarios. The lack of sequence similarity between the multiple effectors that induce RIN4 degradation suggests convergent evolution to modify the same plant targets. Alternatively, RIN4 may function primarily as a sentinel for proteolytic activities from the pathogen or host. Such a role would allow the protection of a large number of proteins and explain the conservation of the cleavage site in diverse species.

Our data suggest several avenues for future research. It will be interesting to determine whether the Pto/Prf pathway is primarily involved in PAMP-triggered resistance or in resistance resulting from the detection of specific effectors. Further characterization

of the proteolytic pathway leading to degradation of RIN4 by the proteasome is a high priority. Also, it will be intriguing to determine whether RIN4 is targeted by effectors from other types of pathogens, such as fungi and oomycetes. In addition, it will be important to test whether other plant resistance-signaling components are also points of vulnerability that are targeted by multiple effectors.

## METHODS

### Plasmids and Cloning Procedures

The *RIN4* homologs from tomato (*Solanum lycopersicum*; Sl *RIN4*) and *Arabidopsis thaliana* (At *RIN4*) were amplified from cDNA by RT-PCR (Advantage 2 RT-PCR kit; Clontech) using the primers described in Supplemental Table 1 online and cloned into Gateway entry vector pDONR207 (Invitrogen). The primers for At *RIN4* were based on At3G25070. The primers for Sl *RIN4* were based on the Tomato Gene Index (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tomato>) SIGI TC174419; this was the only *RIN4* homolog available in the databases at the time the experiments were conducted. The *RIN4* homologs were transferred into the Gateway-compatible *Agrobacterium tumefaciens* binary vector pGWB15 to provide expression in planta with an N-terminal HA tag (courtesy of T. Nakagawa). Similar HA-tagged versions of At *RIN4* had previously been shown to be functional (Day et al., 2005). Recently, the sequence of a second *RIN4* homolog (TC18672) became available from tomato. The two tomato sequences are equally similar to At *RIN4*. This second sequence has the conserved cleavage sites but has not been studied further. The bacterial effector genes, *avrRpt2*, *avrPtoB*, *avrPto*, and *avrB*, were cloned into Gateway binary vector pCB302-3 (Xiang et al., 1999) and transformed into *A. tumefaciens* strain C58C1. For yeast two-hybrid assays, effectors and plant genes were transferred into Gateway-compatible yeast two-hybrid vector pSLR4 as bait and pSLR3 as prey. To generate 3HA:AvrPtoB(deltaC) and AvrPto:3HA, AvrPtoB(deltaC) and AvrPto were cloned into Gateway-compatible binary vectors pGWB15 and pGWB14 with three HA tags at their N or C termini, respectively. To generate 3FLAG:SIRIN4, three FLAG tags were fused onto Sl *RIN4* using primer 3FLAG+SIRIN4F (see Supplemental Table 1 online). To generate HA-RCS-GFP, the cleavage site from RIN4 was first introduced next to GFP using the primers Cyscleave +GFPF and GFPR and then the recombination sites for the Gateway system were added using CyscleaveF and GFPR (see Supplemental Table 1 online for primer sequences). The codons encoding the eight amino acids of the cleavage site are underlined in Supplemental Table 1 online. HA-RCS\*-GFP was generated using the same strategy but with altered forward primers that encoded a mutated cleavage site (mutated codons are indicated in bold in Supplemental Table 1 online). All the clones that were used in this article are listed in Supplemental Table 3 online.

### RNA Isolation and Real-Time PCR

Leaf tissues were collected from tomato plants at 0, 6, 12, and 24 h after challenge with either *Pseudomonas syringae* pv *tomato* T1 or *P. syringae* pv *syringae* B728A. Total RNA was extracted using Plant RNeasy mini kits (Qiagen) and treated with RNase-free DNase (Sigma-Aldrich). First-strand cDNA was synthesized using 1  $\mu$ g of total RNA, oligo(dT) primer, and MMLV reverse transcriptase (Clontech). Real-time PCR was performed using SyBr-Green master mix (Sigma-Aldrich) on a DNA Engine Opticon 2 quantitative real-time PCR machine (Bio-Rad). The results were analyzed by Opticon software provided by Bio-Rad. The data were normalized using the gene encoding  $\alpha$ -tubulin (TC170178) as the

reference gene. The oligonucleotide primers used are listed in Supplemental Table 2 online.

### A. *tumefaciens*-Mediated Transient Assays

*A. tumefaciens* strain C58C1 carrying RIN4 homologs or virulence effectors in the binary vector were grown overnight in Luria-Bertani liquid medium containing 50  $\mu\text{g}/\text{mL}$  kanamycin and 5  $\mu\text{g}/\text{mL}$  tetracycline. Cells were resuspended in infiltration medium (10 mM MES, pH 5.6, 10 mM  $\text{MgCl}_2$ , and 150  $\mu\text{M}$  acetosyringone) to a total concentration of  $\text{OD}_{600}$  of 1.0 and infiltrated into leaves of *Nicotiana benthamiana* as previously described (Wroblewski et al., 2005). For transient assays of tomato, a total concentration of  $\text{OD}_{600}$  of 0.5 was used to avoid a necrotic reaction to *A. tumefaciens* C58C1 (Wroblewski et al., 2005). Infiltrated plants were kept in the laboratory until assayed.

RG-76R, RG-pto11, and RG-prf3 tomato leaves were coinoculated with *P. syringae* pv *tomato* strain T1 at  $\text{OD}_{600} = 10^{-3}$  and *A. tumefaciens* strain C58C1 carrying SI *RIN4* at  $\text{OD}_{600} = 0.5$ . Leaves of transgenic *N. benthamiana* expressing SI *Pto* were coinoculated with *P. syringae* pv *tomato* DC3000, a knockout derivative of *P. syringae* pv *tomato* DC3000 lacking *avrPto*, or a T3SS-deficient mutant of the same strain (at  $\text{OD}_{600} = 10^{-3}$ ) and *A. tumefaciens* strain C58C1 (at  $\text{OD}_{600} = 1.0$ ) carrying SI *RIN4* expressed from the DEX-inducible promoter (Aoyama and Chua, 1997). After 24 h, expression of SI *RIN4* was induced by applying 10 mM DEX (Sigma-Aldrich) on the plant leaves.

Leaves of transgenic *N. benthamiana* that expressed *Pto* were coinoculated with a strain of *Pseudomonas fluorescens* 55 (pHIR11) ( $\text{OD}_{600} = 0.1$ ) expressing or not expressing *avrPto* and *A. tumefaciens* ( $\text{OD}_{600} = 1.0$ ) carrying SI *RIN4* or *GFP* (as a control). After 24 h, leaf samples were harvested and immunoblotted using anti-HA or anti-GFP antibodies (see below). The mock treatment was infiltration with 10 mM  $\text{MgCl}_2$  solution.

For testing the HR of *P. fluorescens* in *N. benthamiana* expressing *Pto*, leaves of the transgenic *N. benthamiana* plants were inoculated with individual strains ( $\text{OD}_{600} = 1.0$ ) of *P. fluorescens* 55 (pHIR11) expressing or not expressing *avrPto*. Only *P. fluorescens* expressing *avrPto* elicited a HR 48 h after the inoculation.

For proteasome and protease inhibitor treatments, leaves of transgenic *N. benthamiana* expressing *Pto* were coinfiltrated with *A. tumefaciens* C58C1 carrying pTA7002-*avrPto* and SI *RIN4*. One day after inoculation, aqueous solutions of 100  $\mu\text{M}$  MG132 or 50  $\mu\text{M}$  protease inhibitor cocktail (Sigma-Aldrich) were infiltrated into the leaves. After an additional 2 h, expression of *AvrPto* was induced by spraying the leaves with 30  $\mu\text{M}$  DEX solution. Four hours after induction, the samples were harvested for immunoblotting as described below.

### Protein Assays

Plant leaves were harvested at the point of incipient chlorosis, prior to any macroscopic necrosis. This was typically 24 h after inoculation or infiltration but varied from 16 to 24 h after inoculation or infiltration depending on the genes being expressed, the age of the plant, and the temperature. Samples were ground into powder in liquid nitrogen. The ground tissues were resuspended in extraction buffer (100 mM Na-phosphate, pH 7.0, 5 mM DTT, and 1% SDS) and boiled for 10 min. Samples were centrifuged at 14,000 rpm for 15 min. The protein concentration in the supernatant was measured using the BCA assay (Pierce). Eight micrograms of total protein was loaded into a mini SDS-PAGE gel (Invitrogen) and transferred to Immobilon-P membrane (Millipore) according to the manufacturer's protocols. Each membrane was hybridized with HA-tag antibody (Convance) or GFP antibody (Convance) to detect the presence of RIN4 protein or GFP protein, respectively. Dilutions of HA-tag primary and secondary antibodies were 1:1000 and 1:10,000, respectively. Dilutions of GFP primary and secondary antibodies were 1:2000 and 1:5000, respectively.

### Yeast Two-Hybrid Assays

Yeast two-hybrid assays used yeast strains EGY48 and YM4721 as described previously (Finley and Brent, 1994). Interactions were tested using SI *RIN4*, At *RIN4*, and *AvrPto* expressed from the LexA bait vector pSLR3 and other components expressed from the prey vector pSLR4 following standard protocols for the LexA system (Clontech).

### Immunoprecipitation

*N. benthamiana* tissues for immunoprecipitation were harvested at least 22 h after infiltration of *Agrobacterium* solutions for *A. tumefaciens*-mediated transient expression of the target protein. Harvested tissues were ground to a powder in liquid nitrogen. Ground samples were resuspended in IP buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 5 mM DTT, and 1 $\times$  Complete Protease Inhibitor [Roche]). The crude lysates were centrifuged at 14,000 rpm for 20 min at 4°C. The protein concentration in each sample was measured by the BCA assay, and equal amounts of each supernatant were used for immunoprecipitation. Samples were incubated with end-over-end agitation with 25  $\mu\text{L}$  anti-HA beads (Roche) for 3 h at 4°C in a spin column (Sigma-Aldrich). Immuno-complexes were washed three times in washing buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, and 1% Triton X-100) and then resuspended in 25  $\mu\text{L}$  of sample buffer (Invitrogen), boiled for 5 min, pelleted, and loaded onto SDS-PAGE gels.

### Pathogenicity Assays

*P. syringae* pv *tomato* strain T1, carrying *avrPto* or the empty vector, was assayed for growth in both tomato RG-76R and a transformant of RG-76R with reduced levels of RIN4 due to RNAi. After inoculation, the plants were kept in 80% humidity and at 28°C in a growth chamber. Three leaf discs from five plants of each genotype were removed 4 h after vacuum-inoculation (day 0) using a 2-cm-diameter cork borer and ground in 10 mM  $\text{MgCl}_2$ ; three leaf discs were removed from each plant at day 2 and at day 4 after inoculation and treated similarly. Serial dilutions were plated on KB agar plates (20 g/L DIFCO protease peptone No.3 [BD], 1.5 g/L  $\text{K}_2\text{HPO}_4$ , 1.6 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 10 mL/L glycerol, pH 7.2) containing 25  $\mu\text{g}/\text{mL}$  rifampicin and 25  $\mu\text{g}/\text{mL}$  kanamycin. Bacterial colonies were incubated at 28°C for 2 d and then colonies were counted.

For measuring the transcription level of *RIN4* in response to host or nonhost pathogen, plants were inoculated with *P. syringae* pv *tomato* T1 or *P. syringae* pv *syringae* B728A at concentrations of  $\text{OD}_{600} = 1.0$ . Leaf tissues were collected at different time points and prepared for real-time PCR (see above).

### Nonspecific Elicitation of HR

To elicit the HR independently of the *Pto* pathway, we transiently overexpressed the *Arabidopsis* resistance gene *RPP8* from the 35S promoter. In *A. tumefaciens*-mediated transient expression assays, *RPP8* expression elicited an HR similar in magnitude and timing to that elicited by the *Pto/AvrPto* interaction. After 48 h, total protein was extracted from the infiltrated tissue and hybridized to the HA-tag antibody.

### Generation of Stable Transgenic Plants

We attempted to generate transgenic tomato plants that either overexpressed *RIN4* in the sense orientation or expressed an RNAi construct containing a fragment of *RIN4*. The overexpressing transgenic plants were generated using vector pGWB15 (T. Nakagawa, Research Institute of Molecular Genetics, Shimane University, Japan). RNAi constructs were made by cloning a fragment of SI *RIN4* (1 to 500 bp from the initial ATG) into pGollum (T. Wroblewski, unpublished data) behind the CaMV 35S

promoter. This vector results in transcription of an inverted repeat of the target gene in concert with a fragment of the  $\beta$ -glucuronidase (*GUS*) reporter gene. This allows the degree of silencing to be monitored using transient expression with *A. tumefaciens* carrying a CaMV 35S:*GUS* gene (Wroblewski et al., 2005). Cocultivation of tomato with *A. tumefaciens* to generate transgenic plants was performed at the UC Davis Plant Transformation Facility (<http://ucdptf.ucdavis.edu/index.shtml>). Only one transgenic plant that overexpressed *RIN4* using pGWB15 was obtained despite multiple attempts in which other genes were simultaneously successfully introduced. The frequency of obtaining transgenic plants containing the *RIN4* RNAi construct was also markedly reduced compared with other constructs introduced at the same time, and only 10 independent transgenics were obtained. Silencing in T1 and T2 plants was assayed by assessing the level of *A. tumefaciens*-mediated transient expression of *GUS* (Wroblewski et al., 2005). The 10 T1 plants varied in their levels of expression of *RIN4* as measured by quantitative real-time PCR; some had nearly wild-type levels; in others, levels were variously reduced to up to 90% less than wild type. T1 plants were self-pollinated to generate sufficient T2 material for analysis. Four T2 lines from two original T1 plants that exhibited at least 85% suppression of *RIN4* expression were available for pathogenicity assays of *P. syringae* pv *tomato* T1 with AvrPto. Three T2 lines from one transgenic line were available for pathogenicity assays of *P. syringae* pv *tomato* T1 with empty vector.

#### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT3G25070 (*At RIN4*), TC174419 (*Sl RIN4*), L20425 (*AvrPto*), AY074795 (*AvrPtoB*), AE016858 (*HopQ1-1*), AY208298 (*HopAm1*), TC170178 (*Sl  $\alpha$ -tubulin*), AAF76306 (*Sl Pto*), and AF220602 (*Sl Prf*).

#### Supplemental Data

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

**Supplemental Figure 1.** The Amino Acid Sequence Alignment of *At RIN4* and *Sl RIN4*.

**Supplemental Figure 2.** Real-Time PCR Analysis Showing the Similar Expression Level of *Sl RIN4* and the Housekeeping Gene Encoding  $\alpha$ -Tubulin.

**Supplemental Figure 3.** Enhanced Transcription of *Sl RIN4* after Challenge with Virulent or Avirulent Strains.

**Supplemental Figure 4.** Protein-Protein Interactions in Yeast and in Planta.

**Supplemental Figure 5.** Degradation of *RIN4* by AvrRpt2 in *N. benthamiana*.

**Supplemental Figure 6.** Degradation of *RIN4* Was Triggered by Two Bacteria Effectors, AvrPto and AvrPtoB, and an Autoactive Pto Mutant and Was Dependent on Pto and Prf.

**Supplemental Table 1.** Oligonucleotide Primers Used for Molecular Constructions.

**Supplemental Table 2.** Primers Used for Quantitative PCR Analysis of *Sl RIN4* Expression in Transgenic Plants.

**Supplemental Table 3.** The List of Constructs in This Article.

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