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Arabidopsis RIN4 Is a Target of the Type III Virulence Effector AvrRpt2 and Modulates RPS2-Mediated Resistance

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Summary

Type III pili deliver effector proteins (virulence factors) from bacterial pathogens to host cells. Plants express disease resistance (R) proteins that respond specifically to a particular type III effector by activating immune responses. We demonstrated previously that two unrelated type III effectors from *Pseudomonas syringae* target and modify the *Arabidopsis* RIN4 protein. Here, we show that AvrRpt2, a third, unrelated type III effector, also targets RIN4 and induces its post-transcriptional disappearance. This effect is independent of the presence of RPS2, the *Arabidopsis* R protein that senses AvrRpt2. RIN4 overexpression inhibits multiple phenotypes associated with AvrRpt2 function. Conversely, disruption of *RIN4* results in RPS2-dependent lethality. RPS2 and RIN4 physically associate in the plant. We suggest that RIN4 is the target of the AvrRpt2 virulence function, and that perturbation of RIN4 activates RPS2. Thus, RIN4 is a point of convergence for the activity of at least three unrelated *P. syringae* type III effectors.

Introduction

Plants express a sophisticated molecular system for recognition of and response to many would be pathogens. In the case of gram-negative bacterial phytopathogens, resistance is triggered when the plant detects a pathogen encoded type III disease effector protein. Plant resistance is often determined by a particular allele of a resistance (*R*) gene. R proteins of the predominant class contain a nucleotide binding site (NB) and leucine-rich repeats (LRR) and are termed NB-LRR proteins. They are intracellular and have the same general domain architecture as mammalian Nod proteins. Plants expressing the correct R protein perceive the presence of a particular type III effector and induce the complex suite of cellular and molecular events comprising the plant defense response (reviewed by Dangl and Jones, 2001). The sum of these responses causes localized cell death around the site of infection termed the hypersensitive response (HR) and restriction of pathogen growth.

The type III effector proteins are delivered via the type III secretion system into the host cell (Collmer et al., 2000; Gálan and Collmer, 1999; Hueck, 1998). An obvious question is: why would bacteria inject proteins that induce resistance into the host cell? In fact, during infection of susceptible (*r*) hosts, many of the type III effector proteins make demonstrable contributions to full bacterial virulence (Chang et al., 2000; Kearney and Staskawicz, 1990; Lorang et al., 1994; Ritter and Dangl, 1995; Shan et al., 2000). In these cases, the effector carries out its virulence function, presumably by suppressing host defenses and/or facilitating nutrient acquisition by the extracellular colony growing in the plant apoplast. There is general interest in understanding the molecular functions of type III effector proteins from phytopathogenic bacterial such as *Pseudomonas syringae* (recently reviewed by Collmer et al., 2002; Nimchuk et al., 2001).

Despite the specificity of R genes for particular pathogen effectors, it has been difficult to detect molecular interaction between R proteins and the type III effectors that trigger their action. Additionally, it seems unlikely that the ∼175 NB-LRR proteins and protein fragments encoded in the *Arabidopsis* genome are a sufficient recognition repertoire to mediate direct recognition of the virulence factors from all possible pathogens, from viruses to fungi, nematodes and aphids, each of which is detected by NB-LRR proteins (The *Arabidopsis* Genome Initiative, 2000).

To accommodate this limited repertoire problem, it was postulated that R proteins might "guard" a limited set of key cellular targets of pathogen virulence factors (Bonas and Lahaye, 2002; Dangl and Jones, 2001; van der Biezen and Jones, 1998). According to the guard hypothesis, a type III effector protein manipulates a host target in order to enhance pathogen virulence. The corresponding R protein monitors the integrity of that particular host target in order to detect manipulation of it by the type III effector. Detection leads to R activation and the induction of downstream defense responses. Thus, the R protein "guards" the plant against pathogens wielding that particular effector, or, in principle, any other virulence factor that similarly manipulates the same target. Thus, although fungal, bacterial, and animal pathogens of plants might have varying life histories, they may all have evolved means to manipulate a limited set of critical host protein complexes. Therefore, the ∼175 NB-LRR gene repertoire of *Arabidopsis* may reflect the upper limit of the number of critical host cellular targets to be guarded. Support for the guard hypothesis is provided by several recent publications (Kim et al., 2002; Kruger et al., 2002; Mackey et al., 2002; Shao et al., 2002; reviewed by Schneider, 2002).

There are two logical extensions of the guard hypothe-

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sis that are germane to our work (Dangl and Jones, 2001). First, a given R protein could, in principle, respond to the presence of two or more unrelated type III effectors that presumably are targeting the same host machinery. We demonstrated that this is true for the RPM1 protein. RPM1 is an NB-LRR protein from *Arabidopsis* and it confers resistance against *Pseudomonas syringae* expressing either of two sequence unrelated effector genes, *avrRpm1* or *avrB* (Grant et al., 1995). When bacteria expressing either of these effector genes are inoculated onto plants expressing RPM1, defenses are induced. However, in *rpm1* plants, AvrRpm1 can enhance pathogen growth (Ritter and Dangl, 1995). RPM1 is a peripheral plasma membrane protein (Boyes et al., 1998), and AvrRpm1 and AvrB are both directed to the host plasma membrane via myristoylation (Nimchuk et al., 2000). Each effector interacts with, and induces phosphorylation of, the *Arabidopsis* RIN4 protein (Mackey et al., 2002). The activities of AvrRpm1 or AvrB that lead to activated resistance or enhanced susceptibility may be one and the same. In *rpm1* plants, this may lead to increased susceptibility by modulating RIN4 activity. RIN4 interacts with RPM1, and manipulation of RIN4 by AvrRpm1 or AvrB could also trigger the activation of defenses induced by RPM1. Alternatively, these molecular phenotypes may be the consequence of the true virulence activities of AvrRpm1 and AvrB. Nevertheless, our data fit well with the hypothesis that RPM1 guards RIN4, a common target of both AvrRpm1 and AvrB.

The second important logical extension of the guard hypothesis is that a host protein complex that is a common target of pathogen virulence functions might be guarded by more than one R protein. Here, we present such an example. RPS2 is an NB-LRR R protein in *Arabidopsis* that induces resistance responses against *P. syringae* expressing the type III effector gene *avrRpt2* (Bent et al., 1994; Mindrinos et al., 1994). AvrRpt2 can enhance bacterial virulence in two distinct circumstances. First, AvrRpt2 can enhance the growth of *P. syringae* pv. *tomato* (Pst DC3000) by approximately one log on No-0(*rps2*) plants, but not on other *rps2* backgrounds (Chen et al., 2000). AvrRpt2 can also enhance bacterial virulence by interfering with RPM1 function (Ritter and Dangl, 1996). The ability of AvrRpt2 to interfere with RPM1 function does not require RPS2.

We tested whether other known *P. syringae* type III effectors also targeted RIN4 and discovered that AvrRpt2 induces its disappearance. AvrPphB and AvrRps4, additional unrelated type III effectors, had no effect on RIN4. The disappearance of RIN4 induced by AvrRpt2 is *RPS2*-independent. Overexpression of RIN4 inhibits three distinct activities of AvrRpt2: (1) its ability to rid the cell of RIN4, (2) its ability to induce RPS2-dependent responses, and (3) its ability to interfere with RPM1 function. Elimination of RIN4 is lethal, and we show that this lethality is RPS2-dependent. We used conditional expression of AvrRpt2 inside the plant cell to demonstrate that it is sufficient to induce the disappearance of RIN4. This disappearance is not regulated at the level of *RIN4* mRNA accumulation. We also show that, in the absence of AvrRpt2, RIN4 and RPS2 physically associate in the plant. Thus, three unrelated type III effectors of *P. syringae*, AvrRpm1, AvrB, and AvrRpt2, all target RIN4 and/or associated factors. We hypothesize that AvrRpt2 targets host defense machinery containing RIN4, and that RPS2 "guards" that machinery to protect the plant against pathogens that manipulate it. Our data complement those of Axtell and Staskawicz (2003 [this issue of *Cell*]), who also localized RPS2 and AvrRpt2 to the plasma membrane, the same subcellular fraction as RIN4, RPM1, AvrRpm1, and AvrB.

Results

AvrRpt2 Induces Disappearance of RIN4

We tested the effect of several type III effectors on RIN4 (Figure 1A). High levels of Pst DC3000 expressing *avrRpt2*, *avrRps4*, or *avrPphB* were infiltrated into leaves of resistant (Col-0) or susceptible *Arabidopsis* (*rps2*, RLD, or *rps5*, respectively; see Experimental Procedures for allele designations of *R* mutants used). AvrRpt2 induced disappearance of RIN4, such that by eight hours after infiltration it was undetectable. AvrRps4 and AvrPphB had no apparent affect on RIN4. The disappearance of RIN4 induced by AvrRpt2 occurred in both *RPS2* and *rps2*. The mutation in the *rps2-101c* allele (used throughout) introduces a stop codon at amino acid 235 of RPS2 and is a presumed null. Therefore, the disappearance of RIN4 induced by AvrRpt2 is RPS2-independent.

We overexpressed RIN4 in both Col-0 and *rps2* (Figure 1B). We constructed multiple, independent, homozygous lines containing a transgene with the strong viral 35S promoter driving overexpression of *RIN4* with an amino-terminal T7 epitope tag (Mackey et al., 2002). Overexpression of RIN4 has no obvious affect on growth, morphology, or fertility of *Arabidopsis*. Overexpression of RIN4 significantly delayed the disappearance of RIN4 induced by Pst DC3000(*avrRpt2*). In these plants, RIN4 was still detectable 24 hr after treatment (Figure 1B). The delayed disappearance of RIN4 was similar in Col-0 and *rps2*, again indicating that this process is unaffected by RPS2. We previously demonstrated that RIN4 was associated with a microsomal membrane fraction (Mackey et al., 2002). Importantly, the overexpressed RIN4 in these lines was also all localized in the microsomal fraction (data not shown), indicating that the lack of disappearance of RIN4 in these lines is not a consequence of its mislocalization.

Overexpression of RIN4 Inhibits RPS2 Function

In Col-0, infiltration of high levels of Pst DC3000(*avrRpt2*) induced a strong HR that becomes macroscopically apparent by 15 hr postinoculation (Figure 2A). This HR is RPS2 dependent and was not observed in *rps2*. Col-0 plants overexpressing RIN4 made no HR in response to Pst DC3000(*avrRpt2*). Representative leaves are pictured, and below each leaf is the number of leaves that exhibited macroscopic HR out of the total number of leaves infiltrated. On Col-0, Pst DC3000(*avrRpm1*) or Pst DC3000(*avrB*) caused a strong HR that was macroscopically apparent by 5 hr postinoculation. Overexpression of RIN4 did not affect the RPM1-dependent HR (data not shown).

We quantified the effect of RIN4 overexpression on RPS2- and RPM1-dependent HR responses using an

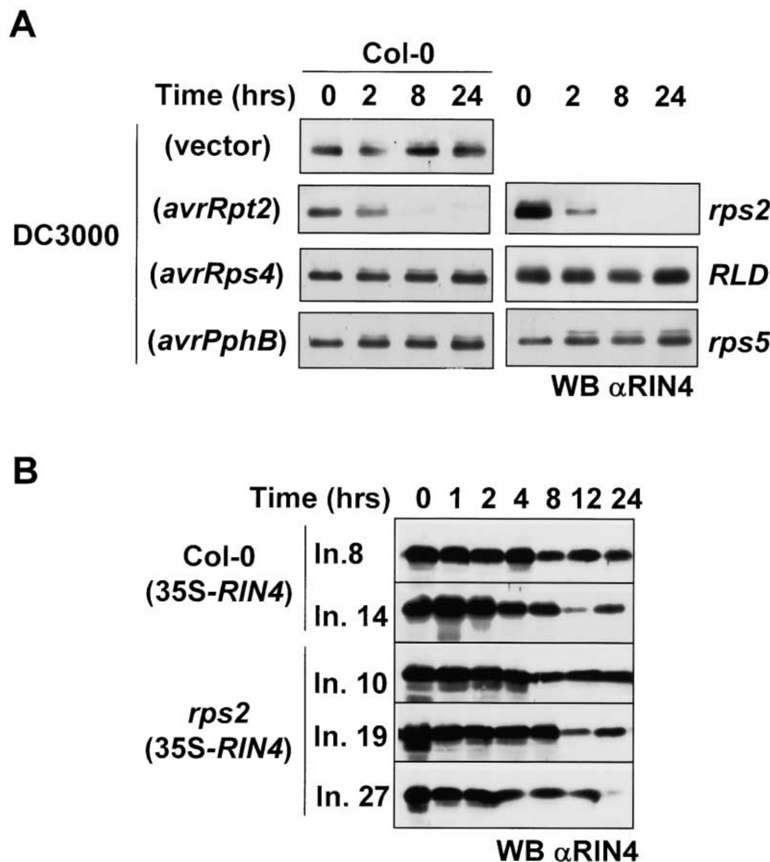

Figure 1. AvrRpt2 Induces Disappearance of RIN4

(A) Col-0 (left column) or *rps2*, RLD, or, *rps5* plants were infiltrated with 5×10^7 cfu/ml of Pst DC3000 carrying empty vector, *avrRpt2*, *avrRps4*, or *avrPphB*. Samples were collected over time and total protein extracts were subjected to an anti-RIN4 Western blot.
(B) Independent lines that contain a 35S-T7tag-RIN4 transgene in Col-0 and *rps2* and express high levels of RIN4 were identified. These lines were analyzed as in (A) with DC3000 expressing *avrRpt2*.

electrolyte leakage assay (Figure 2B). Cell death associated with the HR causes release of electrolytes, which is measured as a change in the conductance of a bath solution (Orlandi et al., 1992). The AvrRpt2-induced HR caused an increase in conductance from Col-0 (relative to *rps2*) that was apparent by 8 hr. Despite the presence of RPS2, this increased conductance was not observed in Col-0 plants overexpressing RIN4. AvrRpm1 or AvrB caused an increase in conductance from Col-0 (relative to *rpm1*), apparent by 4 hr. In Col-0 plants overexpressing RIN4, an initial, RPM1-dependent surge in conductance occurred. The magnitude of this response at later time points was slightly reduced. When the bacteria carry the empty vector, only a slow increase in conductance was induced (note the increase in conductance relative to sample infiltrated with bacteria-free solution). This slow response was unchanged by the RIN4 expression level. Thus, overexpression of RIN4 inhibited the RPS2-dependent HR and may have subtly affected the RPM1-dependent HR.

We also measured the effect of overexpression of RIN4 on RPS2- and RPM1-dependent suppression of bacterial growth (Figure 3). Pst DC3000 carrying the empty vector grew to high levels by four days after infection. This growth was unaffected by the expression level of RIN4 or by the absence of RPM1 and RPS2 (in an *rpm1/rps2* double mutant). When the bacteria expressed *avrRpm1*, growth in Col-0 was significantly reduced relative to that in *rpm1* plants. In Col-0 plants overexpressing RIN4, the RPM1-dependent inhibition of bacterial growth was unaffected. Similarly, when the bacteria expressed *avrRpt2*, growth in Col-0 was significantly reduced relative to that in the *rps2* plants. In Col-0 plants overexpressing RIN4, growth of Pst DC3000(*avrRpt2*) was still significantly reduced relative to that in *rps2*; however, growth was significantly increased relative to that in Col-0. Thus, overexpression of RIN4 partially suppressed the ability of RPS2 to inhibit growth of Pst DC3000(*avrRpt2*).

Elimination of RIN4 Activates RPS2

We previously noted that anti-sense loss of function *rin4* alleles, which eliminated most but not all RIN4 protein, expressed phenotypes reminiscent of plants constitutively activated for defense responses. Based on this finding, we suggested that *RIN4* is formally a negative regulator of basal defense responses (Mackey et al., 2002). A T-DNA insertion into the *RIN4* gene is protein null and seedling lethal (data not shown). We reasoned that this lethality might be the consequence of defense activation very early in development, possibly due to constitutive activation of RPS2 (Mindrinos et al., 1994). If lethality of the *rin4* null is due to ectopic RPS2 activation, then the *rps2* mutation should suppress it. F1 progeny from a *RIN4/rin4* × *rps2-101/rps2-101C* cross were germinated on kanamycin to select for F1s carrying the lethal mutant *rin4* allele. These plants were allowed to self-pollinate. Five of 88 F2 progeny were identified as *rin4* homozygotes by PCR (chi-squared is 1.076; $0.5 > p > 0.1$) and all five were also *rps2-101C* homozygotes (not shown; see Experimental Procedures). The *rps2-*

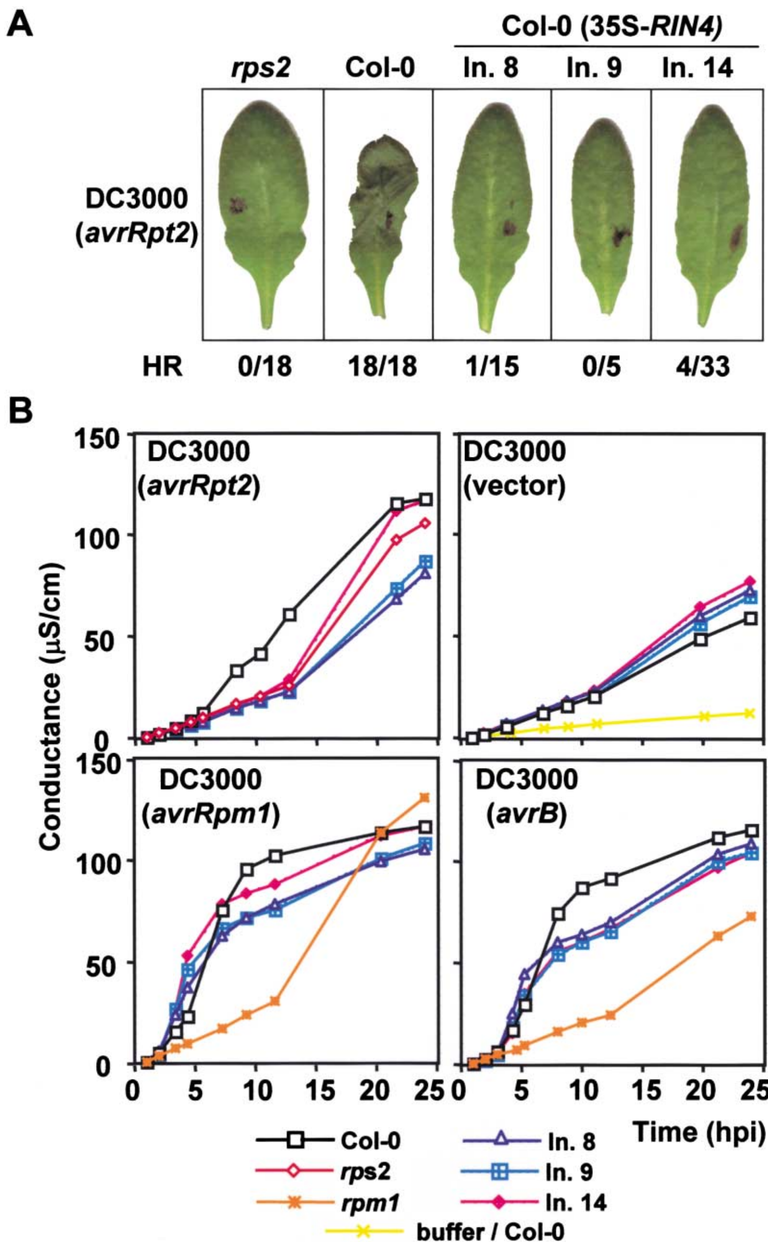

Figure 2. Overexpression of RIN4 Suppresses RPS2-Induced HR

(A) HR phenotypes of *rps2*, Col-0, and three lines of Col-0 that overexpress RIN4 following inoculation with 5×10^7 cfu/ml of Pst DC3000 carrying *avrRpt2*. Representative leaves are shown 20 hr postinoculation (hpi). Beneath each pictured leaf is the number of leaves that showed macroscopic HR over the total number of leaves infiltrated. The black marks are ink.

(B) Ion leakage measurements following inoculation of Pst DC3000 carrying *avrRpt2*, empty vector, *avrRpm1*, or *avrB* into Col-0, *rps2*, *rpm1*, or three independent transgenic lines overexpressing RIN4 in Col-0 (line 8, line 9, and line 14). Infiltration of the 10 mM $MgCl_2$ buffer only into Col-0 is shown along with the data for bacteria carrying the empty vector. This experiment is representative of two independent replicates.

101C mutation segregated freely in this population, as expected. This significant finding indicates that lethality associated with full loss of *RIN4* function is due to constitutive activation of *RPS2*.

RIN4 and RPS2-HA Physically Associate In Vivo

We previously demonstrated that RIN4 associates with RPM1 in vivo (Mackey et al., 2002). We conducted coimmunoprecipitation experiments to test whether RIN4 also interacts with RPS2 (Figure 4). For these experiments, we used transgenic *rps2* plants containing RPS2 with a carboxy-terminal HA tag expressed under the control of its own promoter (gift of M. Axtell and B. Staskawicz). Total protein extracts of these plants were prepared, and immunoprecipitations were conducted with anti-RIN4 sera or control, preimmune sera from the same rabbit. Immunoprecipitated proteins were analyzed by an anti-HA Western blot. RPS2-HA was coprecipitated specifically with the anti-RIN4 sera. The relative amounts of protein in the precipitated and total lanes are not equivalent. The immunoprecipitated protein is overrepresented by 30-fold. Crude fractionation demonstrated that both RPS2 and AvrRpt2 were localized to a microsomal membrane fraction, as predicted given the localization of RIN4 to a similar fraction and as detailed in the accompanying manuscript (data not shown; Axtell and Staskawicz, 2003 [this issue of *Cell*]).

Overexpression of RIN4 Inhibits AvrRpt2 Function

AvrRpt2 can inhibit RPM1 function. When *Arabidopsis* encounters Pst DC3000 expressing both *avrRpm1* and *avrRpt2*, the response of the plant to AvrRpm1 is absent

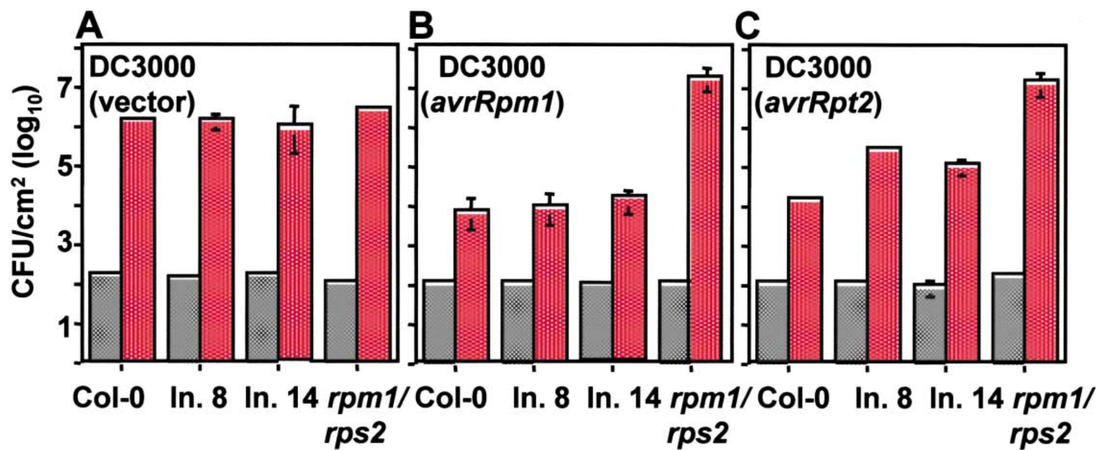

Figure 3. Overexpression of RIN4 Partially Suppresses Inhibition of Bacterial Growth Mediated by RPS2

Growth of Pst DC3000 carrying vector (A), *avrRpm1* (B), or *avrRpt2* (C) was measured on Col-0, two lines overexpressing RIN4 in Col-0, and an *rpm1/rps2* double mutant. Five-week-old plants were infiltrated with 10^4 cfu/ml and the number of bacteria per area of leaf are plotted for day 0 (gray bars) and day 4 (red bars). Error bars represent the standard deviation among three samples and this experiment is representative of four independent replicates. The absence of error bars indicates insignificant differences.

(Ritter and Dangl, 1996). In Col-0, this phenotype is manifested as a shift from an HR in five hours, idiosyncratic to RPM1, to an HR in 15 hr, typical of RPS2. The phenotype is more striking in *rps2* plants, where not only is the RPM1-dependent HR lacking, but there is additionally no RPS2-dependent HR because RPS2 is lacking.

We reproduced this result using three assays, and additionally tested plants overexpressing RIN4. We first quantified using electrolyte leakage, a physiological response associated with the HR (Figure 5A). Pst DC3000(*avrRpm1*) induced a rapid increase in conductance in all plant genotypes except *rpm1/rps2*. Pst DC3000(*avrRpt2*) induced an increase in conductance in Col-0, but not in *rps2*. Pst DC3000(*avrRpm1* + *avrRpt2*) did not induce an increase in conductance in *rpm1/rps2* double or *rps2*. Note that the conductance increases in Col-0 inoculated with either Pst DC3000(*avrRpt2*) or DC3000(*avrRpm1* + *avrRpt2*) were observed at 8–20 hpi, typical of RPS2-dependent HR (black lines, Figure 5A, left and right). Strikingly, the increase in conductance in *rps2*(*35S-RIN4*) induced by DC3000(*avrRpm1* + *avrRpt2*) occurred by 4 hpi, indicative of an RPM1-dependent HR. Thus, AvrRpt2 was unable to interfere with RPM1 function in *rps2* plants that overexpress RIN4. These conductance measurements were supported by qualitative observations of macroscopic HR using the same set of bacterial strain-host genotype combinations (data not shown).

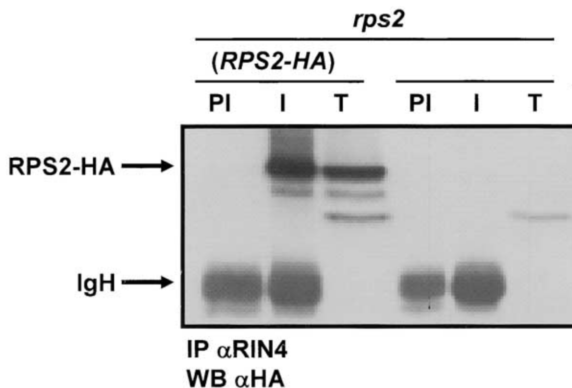

Figure 4. RIN4 Physically Associates with RPS2

Protein from *rps2* and *rps2* expressing *RPS2-HA* under the control of the native *RPS2* promoter was immunoprecipitated with anti-RIN4 sera (I) or with the preimmune sera (PI). Total extracts (T) from *rps2* and *rps2* (*RPS2-HA*) as well as immunoprecipitated samples were analyzed by Western blot with an anti-HA antibody. The relative amounts of protein from the immune pellet and the total extracts are not equivalent. The pellet is over represented by 30-fold. This experiment is representative of two independent replicates.

AvrRpt2 can also inhibit RPM1-mediated disease resistance, as monitored by bacterial growth (Figure 5B). Pst DC3000(*avrRpm1*) grew to high levels on *rpm1/rps2* plants, but did not grow on *RPM1* plants. Two days after infection, Pst DC3000(*avrRpt2*) grew on *rps2* and *RPS2* plants. However, by day 4, bacterial numbers declined in *RPS2* plants yet continued to increase in *rps2* plants. Pst DC3000(*avrRpm1* + *avrRpt2*) grew to high levels on the *rpm1/rps2* double mutant or *rps2*. Growth on *rps2* occurred because AvrRpt2 interferes with RPM1 function (Ritter and Dangl, 1996). On Col-0, growth dropped off by 4 dpi, typical of RPS2-dependent resistance. Thus, in this case, AvrRpt2 inhibited RPM1 function, but growth was still repressed via RPS2 function. Importantly, DC3000(*avrRpm1* + *avrRpt2*) did not grow (even in the first two days) following infection of *rps2*(*35S-RIN4*), demonstrating that AvrRpt2 is unable to inhibit RPM1 function. Thus, as observed for the RPM1-dependent electrolyte leakage in Figure 5A, AvrRpt2 is unable to inhibit RPM1-dependent suppression of bacterial growth in plants overexpressing RIN4.

AvrRpt2 Is Sufficient to Induce Degradation of RIN4

The type III effectors tested in Figure 1 were cloned with a carboxy-terminal HA tag into a dexamethasone (DEX)-

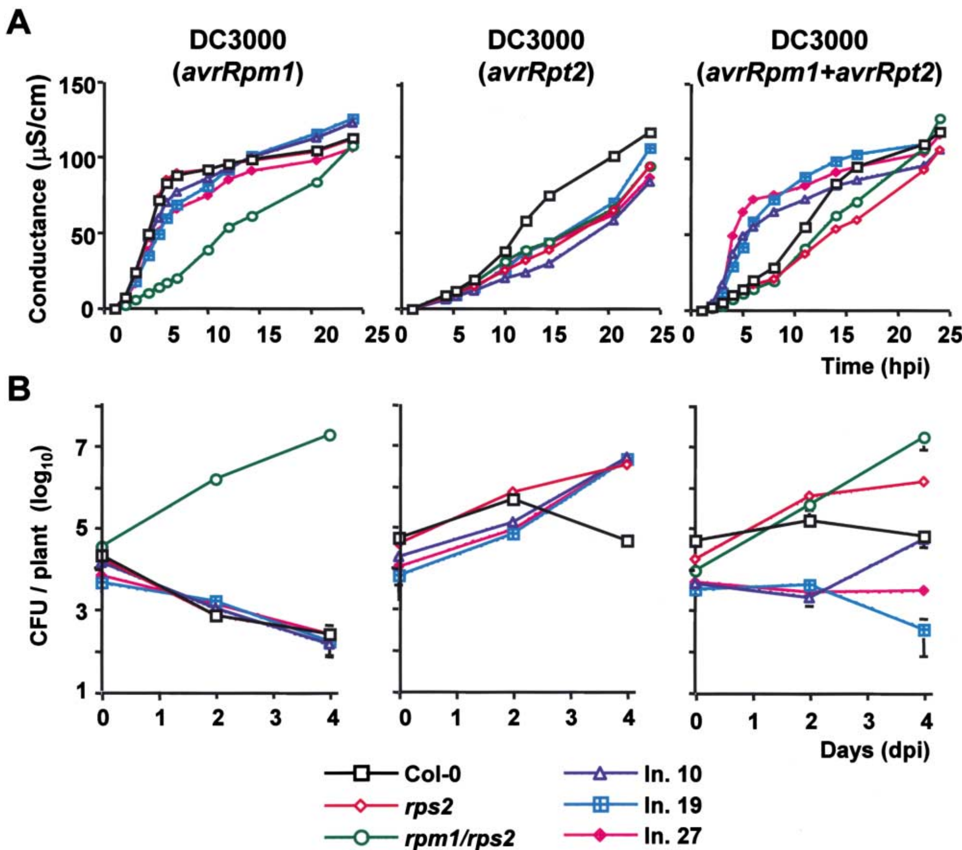

Figure 5. Overexpression of RIN4 Prevents AvrRpt2 from Interfering with RPM1-Dependent Electrolyte Leakage and RPM1-Mediated Disease Resistance

Pst DC3000 carrying *avrRpm1*, *avrRpt2*, or both *avrRpm1* and *avrRpt2* was inoculated into leaves of Col-0, *rps2*, *rpm1/rps2*, or 3 independent transgenic lines overexpressing RIN4 in an *rps2* background (line 10, line 19, and line 27; see code at bottom).

(A) Electrolyte leakage measurements over time following high-density inoculation (see Experimental Procedures). This experiment is representative of two independent replicates.

(B) Bacterial growth measurements following low dose inoculation. Two-week-old plants were dip-inoculated and the number of bacteria per plant were measured over time (Tornero and Dangl, 2001, see Experimental Procedures). This experiment is representative of three independent replicates.

inducible conditional expression system (Aoyama and Chua, 1997). These constructs were transformed into "susceptible" plants lacking functional copies of their respective *R* genes, and homozygous transgenics were established. These plants therefore conditionally express each type III effector but do not make an R-dependent resistance response. Plants that conditionally express AvrRpm1-HA in *rpm1* have been previously described (Mackey et al., 2002); AvrRpm1 induced phosphorylation of RIN4 (seen as reduced mobility of RIN4 in anti-RIN4 Western blot, Figure 6A, upper image; Mackey et al., 2002). Strikingly, DEX treatment resulted in disappearance of RIN4 in total extracts from plants that conditionally expressed AvrRpt2-HA in *rps2*. Note that these plants expressed moderately reduced levels of RIN4 in the absence of dexamethasone, presumably due to "leaky" expression of AvrRpt2-HA. Conditional expression of either AvrRps4-HA or AvrPphB-HA in RLD and *rps5*, respectively, did not detectably affect RIN4. Each of these HA tagged effectors accumulated (seen by anti-HA Western blot, Figure 6A, lower image). The apparent mobility of the HA-tagged fragments of AvrRpt2 (~20 kDa; Mudgett and Staskawicz, 1999; Puri et al., 1997) and AvrPphB (~23 and 25 kDa; Puri et al., 1997) are consistent with previous publications demonstrating that these effectors are proteolytically processed in the plant. Our results for AvrRps4 (~7 kDa) suggest that this protein might also be proteolytically processed in the plant. Each of these HA-tagged effectors elicited HR-like cell death when the transgenes were introduced into "resistant" plants (Col-0, which expresses *RPS4* and *RPS5* in addition to *RPM1* and *RPS2*), indicating that they are all functional. We conclude from this experiment that AvrRpt2 induces disappearance of RIN4, and that it can do so in the absence of any other contributions from Pst DC3000.

In order to determine whether the effect of AvrRpt2 on RIN4 protein levels was mediated by reduced transcription of RIN4, total RNA was prepared from various *rps2* plants and measured via RNA blot (Figure 6B). As expected, plants overexpressing RIN4 expressed elevated levels of RIN4 message at the correct size of ~850 nt. Non-transgenic *rps2* plants, uninduced plants carrying transgenic AvrRpt2-HA, and plants expressing

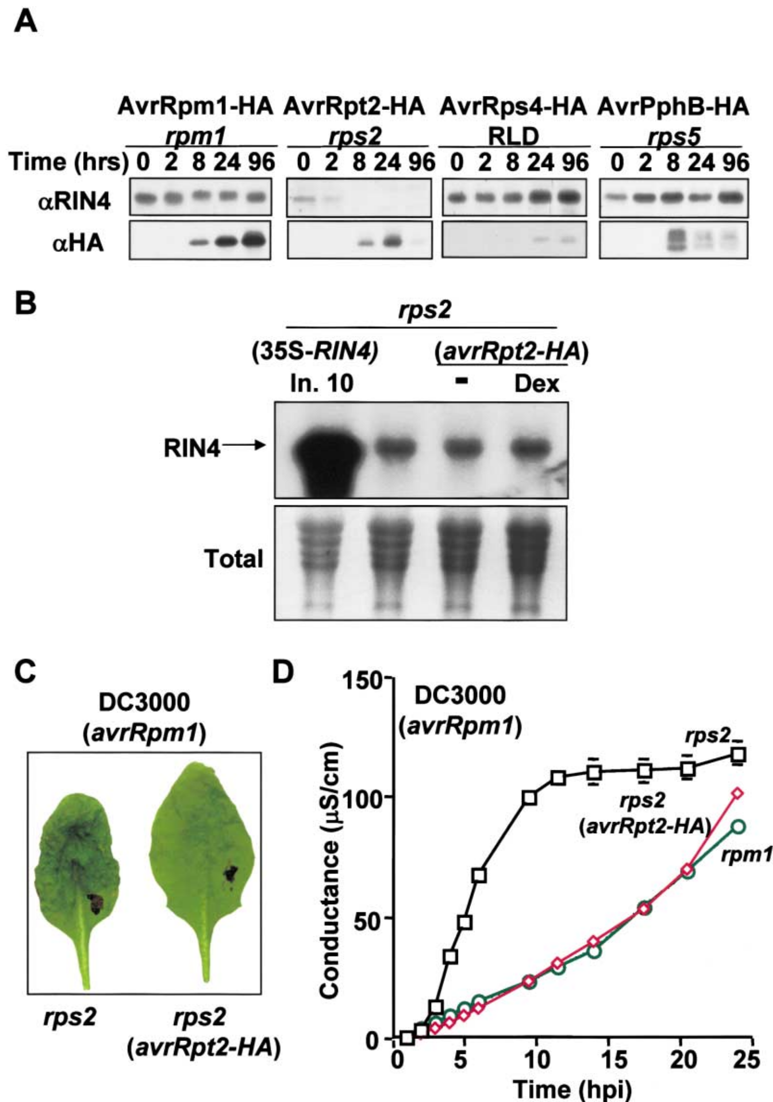

Figure 6. AvrRpt2 Is Sufficient to Induce Degradation of RIN4 and to Interfere with RPM1 Function

(A) Three-week-old transgenic plants expressing dexamethasone inducible *avrRpm1-HA* in *rpm1*, *avrRpt2-HA* in *rps2*, *avrRps4* in RLD, and *avrPphB* in *rps5* were sprayed with DEX (20 μM). Samples were collected over time and total protein extracts were subjected to anti-RIN4 western (upper image) and anti-HA western (lower image). The sizes of the HA tagged fragments are indicated in the text.

(B) Total RNA was prepared from a line overexpressing RIN4 in *rps2*, *rps2*, or *rps2* conditionally expressing *avrRpt2*-HA 8 hr after the plants had been mock treated (−) or sprayed with 20 μM DEX. Samples were subjected to a RIN4 RNA blot (upper image). A negative image of ethidium bromide-stained total RNA is shown (lower image).

(C) HR phenotypes of *rps2* or *rps2* conditionally expressing *avrRpt2-HA* following inoculation with 5 × 10^7 cfu/ml of Pst DC3000(*avrRpm1*). Representative leaves are shown 20 hr postinoculation.

(D) Ion leakage measurements following infiltration of Pst DC3000 carrying *avrRpm1* into *rps2* (red diamonds), *rpm1* (orange asterisks), or DEX *avrRpt2-HA* in *rps2* (black + s). Plants in (C) and (D) were sprayed with 20 μM DEX 8 hr prior to infiltration.

AvrRpt2-HA (8 hr after DEX treatment) all expressed similar levels of the RIN4 message. This was in contrast to the levels of RIN4 protein, which are below detection limits in plants conditionally expressing AvrRpt2-HA. Therefore, AvrRpt2 causes disappearance of RIN4 without affecting *RIN4* transcription.

AvrRpt2 expression is sufficient to inhibit the function of RPM1. Following DEX induction, *rps2* plants expressing AvrRpt2-HA did not initiate an RPM1-dependent HR. This was observed both macroscopically (Figure 6C) and by electrolyte leakage (Figure 6D). In each assay, the response to AvrRpm1 normally mediated by RPM1 was completely absent. A caveat to this experiment is that type III effectors other than AvrRpm1 are also delivered by Pst DC3000. It is possible that these other effectors are required, in combination with AvrRpt2, to interfere with RPM1. However, in plants that express only AvrRpm1 and AvrRpt2 as transgenes, AvrRpt2 still interfered with RPM1 (data not shown). Therefore, we conclude that AvrRpt2 is sufficient to inhibit function of RPM1.

Discussion

We recently demonstrated that two sequence-unrelated type III effector proteins from *P. syringae* target a novel *Arabidopsis* protein, RIN4, inside the host cell at the plasma membrane (Mackey et al., 2002; see Introduction). We presented a model suggesting that RIN4 is both a target for the virulence function of these two type III effector proteins, AvrRpm1 and AvrB, and, at least formally, a negative regulator of basal defense. We proposed that the targeting of RIN4 by the type III effectors is monitored by RPM1, leading to rapid defense activation.

Here, we significantly extend that model and provide evidence that a third, sequence unrelated, *P. syringae* type III effector protein called AvrRpt2 induces the post-transcriptional elimination of RIN4. We propose a model (Figure 7) in which RIN4 is manipulated by the virulence activity of AvrRpt2. Activation of RPS2 results from AvrRpt2-dependent RIN4 elimination, mechanistically explaining our previous formal definition of RIN4 as a

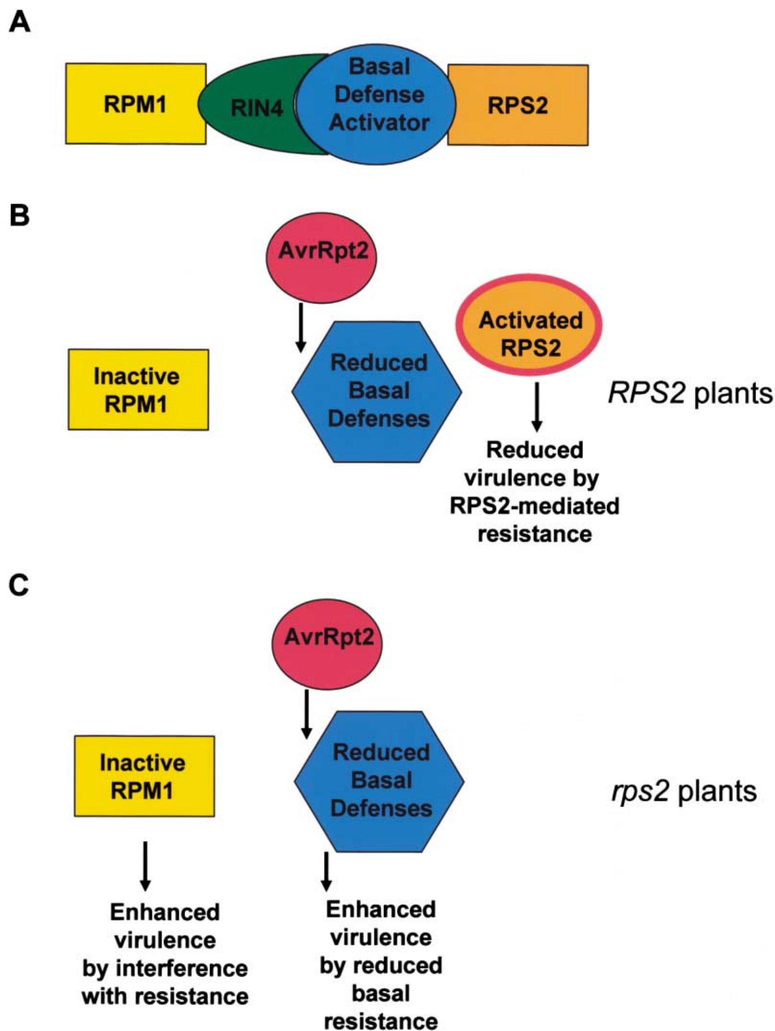

Figure 7. Model: RIN4 Is Part of a Defense Activator that Is Targeted by AvrRpt2 and Guarded by RPS2

(A) RIN4 interacts directly with RPM1 and indirectly with RPS2. The interaction of RIN4 with RPS2 is via a putative activator(s) of defense proposed by Chen et al. (2000).

(B) AvrRpt2 is delivered into the plant cell via type III secretion and targets RIN4. RIN4 elimination results in RPS2 activation in resistant plants.

(C) In *rps2* plants, AvrRpt2 inhibits RPM1 by inducing disappearance of RIN4. AvrRpt2 also perturbs the defense activator in a way that enhances bacterial virulence. Events in (B) and (C) are inhibited by overexpression of RIN4 and constitutively induced by disruption of *RIN4*.

negative regulator of defense. Based on this and the accompanying paper of Axtell and Staskawicz (2003), we propose that RPS2 "guards" the plant against pathogens that use AvrRpt2 to eliminate RIN4.

We propose that the AvrRpt2-induced disappearance of RIN4 activates RPS2. We provide two strong lines of evidence for this proposal: (1) inhibiting RIN4 disappearance prevents RPS2 activation and (2) the lethality associated with *RIN4* disruption requires RPS2. The AvrRpt2-induced disappearance of RIN4 is inhibited by overexpression of RIN4. In this case, AvrRpt2 still causes a diminution in the levels of RIN4, but disappearance is significantly slowed. The disappearance of RIN4 occurs late enough that an "early" read-out (*RPS2*-dependent HR) is completely blocked (Figure 2). On the other hand, the eventual disappearance of RIN4 permits a "late" read-out (*RPS2*-dependent inhibition of bacterial growth) to partially function (Figure 3). Interestingly, when RIN4 levels are low, but still detectable, plants initiate an essentially normal RPS2-dependent response to AvrRpt2 (Mackey et al., 2002). Thus, only by eliminating nearly all, or all, of RIN4 does AvrRpt2 activate RPS2. AvrRpt2-independent disappearance of RIN4, due to disruption of *RIN4*, constitutively activates RPS2 and causes embryo lethality (Collins et al., 1999; Mindrinos et al., 1994; Oldroyd and Staskawicz, 1998; Stokes et al., 2002; Tao et al., 2000). This model explains the activation of basal defenses and age related-lesioning observed previously in plants with reduced levels of RIN4 (Mackey et al., 2002).

We hypothesize that the disappearance of RIN4 induced by AvrRpt2 is specifically coupled to its virulence activity. Three pieces of evidence support this model. First, RIN4 elimination is specific to AvrRpt2; RIN4 is stable in the presence of other type III effectors from *P. syringae* (Figure 1 and Mackey et al., 2002). Thus, RIN4 is not a general target of type III virulence factors from this pathogen. Second, AvrRpt2 eliminates RIN4 in both RPS2 and *rps2* plants. Thus, disappearance of RIN4 is not merely a consequence of activation of RPS2, but rather is actively induced by AvrRpt2 and is genetically upstream of RPS2. Third, the ability of AvrRpt2 to interfere with function of RPM1 (Ritter and Dangl, 1996) relies on the induced disappearance of RIN4. Thus, disappearance of RIN4 is important for this specific virulence activity of AvrRpt2.

In *RPS2* plants, the disappearance of RIN4 induced by AvrRpt2 leads to activation of defenses. In *rps2*

plants, by contrast, disappearance of RIN4 induced by AvrRpt2 leads to the repression of defense responses. AvrRpt2 interferes with RPM1 function by inducing RIN4 disappearance. Chen et al. (2000) demonstrated that AvrRpt2 delays defense responses in No-0(*rps2*) plants, indicating that AvrRpt2 targets a positive regulator of defense. RIN4 may be a component of this positive regulator of defense. AvrRpt2 also induces disappearance of RPM1, but the timing (~15 hpi; Boyes et al., 1998; D.M., J. Nam and J.L.D., unpublished data) is delayed relative to the timing of disappearance of RIN4 (by 3 to 4 hr, data not shown). RPM1 function, measured as an HR, is normally observed by 5 hr. This is subsequent to RIN4 disappearance but prior to RPM1 disappearance. In plants that overexpress RIN4, AvrRpt2-induced disappearance of RIN4 is incomplete by 24 hpi. Thus, RIN4 persists long enough that RPM1 can be activated by AvrRpm1 or AvrB. We suggest that the ability of AvrRpt2 to interfere with RPM1 is a specific manifestation of its more general virulence activity.

Both RPS2 and RPM1 coimmunoprecipitate with RIN4 from *Arabidopsis* extracts (Figure 4). We previously demonstrated a direct physical interaction between RIN4 and the amino terminus of RPM1 (amino acids 1–176). A similar portion of RPS2 (amino acids 1–159) does not interact with RIN4, though this negative result is of limited interpretive utility. We propose that RPS2 interacts indirectly with RIN4, via additional components of the positive defense activator postulated by Chen et al. (2000). AvrRpm1 and AvrB interact with, and induce phosphorylation of, RIN4, presumably perturbing the function of the hypothesized defense activator. RPM1 activation is a consequence of these manipulations of RIN4 (Mackey et al., 2002). Here, we extend this idea to suggest that AvrRpt2 also targets RIN4, perhaps to perturb the same hypothesized defense activator. RPS2 activation is a consequence of the induced disappearance of RIN4. We predict that additional proteins in the RIN4 complex will (1) function to positively regulate defenses and (2) interact directly with RPS2.

Our data significantly extend the "guard hypothesis" for NB-LRR function (Dangl and Jones, 2001) by demonstrating that multiple R proteins, RPM1 and RPS2, can guard the same RIN4-containing cellular machinery. RPM1, RPS2, and RIN4 probably exist as part of one large complex. This provides an explanation for the previously perplexing finding that RPS2 can associate with AvrB in vivo (Leister and Katagiri, 2000). These well-studied R proteins and the bacterial effectors against which they "guard" comprise only a small sampling of the diversity present in plants and bacteria. It would be thus be unsurprising to find additional NB-LRR proteins associated with RIN4 and it partners. Similarly, *P. syringae* encodes at least three effectors from bacterial pathogens of diverse hosts, each of which manipulate RIN4 (AvrRpm1, AvrB, and AvrRpt2). This highlights the evolutionary advantage, for the pathogen, of perturbing this particular host system. It would, therefore, not be surprising to find that other pathogens also target RIN4 and its proposed associated proteins.

Additional cases of type III effectors inhibiting plant defense responses have been described. In *P. syringae* pv *phaseolicola*, AvrPphC can block host resistance responses triggered by AvrPphF, which can in turn block resistance responses triggered by as yet undefined Avr proteins (Tsiamis et al., 2000). Similarly, genetic experiments have established that disease effectors of flax rust can inhibit the recognition of another molecule produced by flax rust (Lawrence, 1995; Lawrence et al., 1981). Conflict between the function of pathogen type III effectors and plant R proteins probably results from continual, evolutionary one-upmanship. In the pathogen, an effector evolves that enhances virulence of the pathogen. In the plant, an R protein evolves that can recognize either the action of this effector, or the effector itself, and consequently trigger resistance. In the pathogen, another effector evolves that can inactivate this R protein. Thus, the repertoire of R proteins may guard relatively few protein machines within plant cells, and these machines are, in turn, repeatedly targeted by pathogen virulence factors.

Arabidopsis carries genes encoding ~175 NB-LRR proteins; some of these are truncated and presumably do not specify resistance. Thus, the repertoire of possible sentinels in the plant is limited. Given the large number of possible effectors from just a single strain of one pathogen, the fully sequenced *P. syringae* DC3000 genome (Collmer et al., 2002), it is unlikely that this limited repertoire of R proteins is sufficient to mediate direct recognition of all the possible virulence factors from all the possible pathogens in the *Arabidopsis* environment. One solution to this conundrum is that virulence factors of all pathogens, from viruses to bacteria, from fungi to aphids, target a limited set of host machines that are important in defense responses and/or nutrient acquisition. These factors are met at their target by the guards of cellular homeostasis, the NB-LRR proteins of the plant immune system. There are clear parallels in this model with emerging concepts of type III effector manipulation of animal cell signaling (Staskawicz et al., 2001). Continuing inter-kingdom comparisons will advance our understanding of innate immune responses.

Experimental Procedures

Protein

Total protein extracts were prepared by grinding approximately 3 square centimeters of leaf tissue in 100 μl of grinding buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM DTT, and plant protease inhibitor cocktail [Sigma-Aldrich]) and pelleting insoluble debris by centrifugation at 20,000 × g for 10 min at 4°C. Concentration of soluble protein was determined with the Bio-Rad protein assay (Bio-Rad). Samples were separated on SDS-PAGE gels (mini protean, Bio-Rad) of 12% (or 7.5% for RPS2-HA) and transferred to nitrocellulose. Western blots were done by standard methods. Anti-RIN4 sera (Mackey et al., 2002) was used at a dilution of 1:5000. Detection of HA was with the 3F10 monoclonal anti-HA antibody (Roche).

For immunoprecipitations, tissue was first ground in liquid nitrogen with a mortar and pestle. This material was then homogenized (Polytron, Kinematica) in 2 ml of buffer (50 mM HEPES [pH 7.4], 50 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, and plant protease inhibitor cocktail [Sigma-Aldrich]) per 1 g of tissue. Insoluble material was pelleted by centrifugation at 20,000 × g for 20 min at 4°C. An amount totaling 1.5 ml of this supernatant was first precleared by adding 100 μl of a 1/1 mix of protein A- and protein G-agarose (Boehringer Mannheim) and incubated at 4°C for 10 min on an orbital shaker. The cleared supernatant was then removed and combined with 5 μl with either the preimmune or the anti-RIN4 sera. After incubation at 4°C for 2 hr, 50 μl of a 1/1 mix of protein A- and protein

G-agarose was added and the reaction was rolled at 4°C for 6 hr. The beads were washed 3 times in 1.5 ml of the same buffer (except 0.1% Triton X-100 instead of 0.2%).

Plants

The following plant genotypes were used in this work: *rps2-101C* in an allele of *RPS2* in Col-0 with a stop codon following amino acid 235 (Mindrinos et al., 1994); *rpm1-3* is an allele of *RPM1* with a stop codon following amino acid 87 (Grant et al., 1995); RLD is an accession of *Arabidopsis* lacking *RPS4* (Gassmann et al., 1999); *rps5-2* is an allele of *RPS5* with a proline to serine exchange at amino acid 799, within the LRRs (Warren et al., 1998).

Transgenic Plants

The plasmid for overexpression of *RIN4* has been previously described (Mackey et al., 2002). To inducibly express of AvrRpt2, AvrRps4, and AvrPphB in planta, each gene with a carboxy-terminal HA-tag was cloned into pTA7002 (Aoyama and Chua, 1997). Transgenic plants were generated by vacuum infiltrating *Agrobacterium tumerifaciens* (GV3101) carrying these plasmids into flowering *Arabidopsis* (Clough and Bent, 1998). Transgenic progeny were selected by spraying with BASTA (AgrEvo) (for pBAR-35S-T7-RIN4) or growth on plates of Gamborg's B5 (Gibco) with 20 μM Hygromycin B (Sigma) (for derivatives of pTA7002). Transgenic lines with single insertion loci were identified and carried to homozygosity.

Double-Mutant Construction

DNA from 88 F2s was extracted by conventional methods. DNA samples were used in PCR reactions for identification of F2 individuals homozygous for *rin4* and *rps2-101C*. The ORF of *RIN4* was amplified with the following primers: sense: 5′-GCA-CGT-TCG-AAT-GTA-CC-3′; anti-sense: 5′-AAT-TCT-CAT-TTT-CCT-CCA-AAG-CC. For *rps2-101C*, a PCR-CAPS marker was amplified with the primers corresponding to nucleotides 537–557 (5′-TGT-TTA-TGG-ACC-TGG-TGG GGT-3′) and 955–935 (5′-TCT-TTG-TGC-GCA-CCC-TCG-ACA-3′) of the *RPS2* sequence. The PCR products were digested with DdeI, which cuts the mutants DNA into a doublet. Both PCR reactions were performed in the following buffer 50 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM MgCl2, 1% Triton X-100, 0.2 μM of each primers and 0.20 mM of each dNTPs, and the following conditions: an initial denaturation step of 2 min at 94°C, followed by 40 cycles of 10 s at 94°C, 1 min at 55°C, and 2 min at 72°C. A final elongation step of 10 min at 72°C was added. The PCR products were resolved on 1% and 4% agarose gel for RIN4 and the *RPS2* CAPS marker, respectively

Pseudomonas syringae

DC3000 carrying either pVSP61 or derivatives of this plasmid containing *avr* genes have been described (Bisgrove et al., 1994; Grant et al., 1995). For HR assays, ion leakage assays, and assays of RIN4 status, DC3000 was resuspended at 5×10^7 cfu/mL in 10 mM $MgCl_2$. Bacteria were infiltrated into leaves of 4–5 week-old plants.

For measurements of ion leakage, 8 leaf discs (8 mM diameter) were removed immediately following infiltration ($t = 0$) and floated in 50 ml of water. After thirty minutes, the wash water was removed and replaced with 10 ml of fresh water. Conductance of this water was then measured over time.

Bacterial growth in plant leaves was measured by two methods. Figure 3 was done by inoculating five-week-old plants with 10^4 cfu/ml. Leaf discs were bored from the infiltrated area, ground in 10 mM $mgCl_2$, and serially diluted to measure bacterial numbers. Data reported in Figure 5B was generated essentially as described (Tornero and Dangl, 2001). Two-week-old plants were dip inoculated in DC3000 suspended at 25 million CFU per ml in 10 mM $MgCl_2$ and 0.02% Silwet L-77 (CKWitco Corporation) and kept covered for 24 hr. At one hour after dipping ($t = 0$), day 2, and day 4, the number of CFU per plant was determined. Four plants (whole aerial tissue) were placed in 1 ml of 10 mM $MgCl_2$ and 0.02% Silwet L-77 and shaken at 30°C for one hour. Serial dilutions of the solution were used to titer the bacteria.

RNA Analysis

To prepare RNA, leaves of three-week-old plants were collected 8 hr after mock treatment or treatment with 20 μM dexamethasone (Sigma) and 0.0075% silwet L-77 and ground by mortar and pestle in liquid nitrogen. RNA was extracted with Trizol following the manufacturer's protocol (GibcoBRL). Approximately 15 mg of total RNA was loaded per lane in denaturing gels. RNA was transferred to Hybond-N membranes (Amersham Pharmacia Biotech) and hybridization was performed in ULTRAhyb (Ambion) at 45°C per the manufacturer's directions. The *RIN4* probe was generated by pcr and labeled with α-ATP using reagents provided in the Prime-It II random primer labeling kit (Stratagene).

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