

# RIN4 Interacts with *Pseudomonas syringae* Type III Effector Molecules and Is Required for RPM1-Mediated Resistance in *Arabidopsis*

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## Summary

In *Arabidopsis*, *RPM1* confers resistance against *Pseudomonas syringae* expressing either of two sequence unrelated type III effectors, *AvrRpm1* or *AvrB*. An *RPM1*-interacting protein (*RIN4*) coimmunoprecipitates from plant cell extracts with *AvrB*, *AvrRpm1*, or *RPM1*. Reduction of *RIN4* protein levels inhibits both the hypersensitive response and the restriction of pathogen growth controlled by *RPM1*. *RIN4* reduction causes diminution of *RPM1*. *RIN4* reduction results in heightened resistance to virulent *Peronospora parasitica* and *P. syringae*, and ectopic defense gene expression. Thus, *RIN4* positively regulates *RPM1*-mediated resistance yet is, formally, a negative regulator of basal defense responses. *AvrRpm1* and *AvrB* induce *RIN4* phosphorylation. This may enhance *RIN4* activity as a negative regulator of plant defense, facilitating pathogen growth. *RPM1* may “guard” against pathogens that use *AvrRpm1* and *AvrB* to manipulate *RIN4* activity.

## Introduction

Plants maintain a variety of static defenses, which stymie many would-be pathogens. However, organisms ranging from viruses to insects have evolved mechanisms to successfully plunder photosynthate from plants. To combat these organisms, plants rely on an innate immune response. Central to this response are disease resistance (*R*) genes (Flor, 1971). Products of *R* genes mediate recognition of potential pathogens and initiate a battery of active defense responses (Dangl and Jones, 2001). These responses are layered onto, and genetically intersect with, a basal defense system that can limit the growth of a virulent pathogen even in the absence of *R* function (Feys and Parker, 2000; Glazebrook, 2001; McDowell and Dangl, 2000). *R*-dependent resistance is frequently associated with a programmed cell death around the site of infection termed the hypersensitive response (HR; Morel and Dangl, 1997). A successful resistance response occurs only if the plant has the appropriate *R* allele that conditions recognition of a signal produced by the pathogen. This signal is generally

specified by a single gene of the pathogen, termed an avirulence (*avr*) gene. If plant and pathogen carry matched *R* and *avr* genes, the plant responds and pathogen growth is curtailed. Here, the avirulence genes are appropriately named; they render the pathogen avirulent. However, the avirulence label can be misleading. On plants lacking the appropriate *R* gene, *avr* genes are often required for maximal virulence of a particular pathogen strain.

*Avr* proteins from a variety of phytopathogenic bacteria are biologically active within host plant cells. *Avr* protein function requires the evolutionarily conserved, bacterial type III secretion system. This system can deliver type III effector proteins, including *Avr* proteins, from the bacteria into the host cell (Hueck, 1998). When expressed within plant cells expressing the cognate *R* protein, *Avr* proteins can induce cell death reminiscent of the HR (Gopalan et al., 1996; Leister et al., 1996; Van den Ackerveken et al., 1996; reviewed in Nimchuk et al., 2001). When expressed within plants lacking the cognate *R* protein, some *Avr* proteins can cause responses that resemble disease symptoms (Duan et al., 1999; Gopalan et al., 1996; Nimchuk et al., 2000) and can function in *trans* to enhance virulence (Chen et al., 2000; D.M., J. Chang, and J.L.D., unpublished). These findings establish two important aspects of the action of *Avr* protein as type III effectors: (1) host genotype-dependent responses can be induced by *Avr* proteins within plant cells and (2) both resistance responses and phenotypes reminiscent of disease symptoms can be induced by *Avr* proteins independent of the rest of the pathogen.

*R* proteins and *Avr* proteins can colocalize within the plant cell. *RPM1* is peripherally associated with the plasma membrane (Boyes et al., 1998). Several *Pseudomonas syringae* *Avr* proteins, including *AvrRpm1* and *AvrB*, are similarly localized when expressed in the plant (Nimchuk et al., 2000). Of this type III effector class, at least *AvrB*, *AvrRpm1*, and *AvrPto* require eukaryote-specific myristoylation sites for their membrane localization and function (Nimchuk et al., 2000; Shan et al., 2000). Models in which *Avr* proteins and *R* proteins interact directly have been widely proposed, however, data supporting these models is limited. Two alternative hypotheses are (1) the *R* proteins interact with *Avr* proteins only in the context of a higher-order complex (Leister and Katagiri, 2000), and (2) the elicitors that interact with *R* proteins are the in planta by-products of *Avr* protein activity. Colocalization of *AvrRpm1* and *AvrB* with *RPM1* is consistent with each of these hypotheses.

Most *R* proteins contain leucine-rich repeats (LRRs), that act as probable specificity determinants of pathogen recognition, and a central nucleotide-binding domain (NB). Diversifying selection has acted on amino acids within the LRRs that are predicted to be solvent exposed, based on analogy to crystal structures of LRRs to date (Jones and Jones, 1996; Kajava, 1998). Construction of chimeric *R* proteins strongly supports the idea that the LRRs contribute to specificity (reviewed in Ellis et al., 2000). Additionally, the LRRs encoded by

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*Pi-ta*, a rice *R* gene, interact directly with the AvrPita protein from rice blast fungus (Jia et al., 2000). The LRRs can also specify interaction with other proteins of the plant. Amino acids in the LRRs of RPS2 determine its ability to function in different genetic backgrounds, presumably due to interactions with distinct cofactors in those backgrounds (Banerjee et al., 2001). But the specificity of *R* proteins is perhaps not solely determined by the LRRs. The specificity of flax *L* alleles is also modulated by amino acids outside the LRRs, and diversifying selection has also acted on the amino-terminal domain of *L* proteins (Luck et al., 2000). The amino termini of *L* alleles have similarity to the cytoplasmic domains of *Drosophila* Toll and the interleukin-1 receptor (TIR), and these *R* proteins belong to the TIR-NB-LRR subclass. RPM1 is of another *R* protein class that has a putative coiled-coil (CC) domain instead of the TIR, and it is hence a CC-NB-LRR protein.

RIN4 was identified via its interaction in yeast with AvrB. In plant extracts, RIN4 interacts with RPM1 and with two sequence unrelated Avr proteins (AvrRpm1 and AvrB) that are each recognized by RPM1. RIN4 is required for activation of RPM1-dependent HR and RPM1-dependent inhibition of bacterial growth. RIN4 is also required for accumulation of RPM1, probably explaining these effects. RIN4 can function as a negative regulator of basal plant defense. Reduction of RIN4 levels enhances resistance of *Arabidopsis* to virulent bacterial and oomycete pathogens. In addition to enhancing basal defenses, reduction of RIN4 levels results in constitutive defense gene expression. AvrRpm1 or AvrB, delivered from bacteria or expressed in planta, induce phosphorylation of RIN4. AvrB also induces increased accumulation of RIN4. We hypothesize that AvrRpm1 and AvrB manipulate RIN4 levels and activity to enhance its function as a negative regulator of plant defense. We further suggest that RPM1 “guards” the plant by perceiving the Avr-dependent perturbation of RIN4 and inducing disease resistance.

## Results

### RIN4 Interacts with RPM1, AvrB, and AvrRpm1

We conducted a yeast two-hybrid screen to identify cDNAs from *Arabidopsis* that interacted with AvrB (data not shown). One protein identified in this screen also interacted with RPM1 (data not shown) and we named the gene encoding it *RIN4* (RPM1 interacting protein 4). *RIN1-3* encode proteins that interact with RPM1 in this screen and will be described elsewhere. In yeast, RIN4 interacted strongly with the amino-terminal 176 amino acids (aa) of RPM1 and very weakly with aa 55 to 341 of RPM1. It is possible that aa 1 to 54 (which contain the CC domain) are important for the interaction or that aa 177 to 341 (which contain the NB domain) inhibit the interaction. RIN4 did not interact with baits containing the amino-terminal ends of RPS2 (aa 1 to 159; Bent et al., 1994; Mindrinos et al., 1994) or RPP5 (aa 1 to 233; van der Biezen et al., 2000; data not shown). *RIN4* encodes a 211 aa protein (Figure 1A) with no known motifs and no predicted subcellular localization (but see below).

The carboxy-terminal ~2/3 of RIN4 were produced in *E. coli* and antisera was raised in rabbits. Our antisera

specifically detects RIN4 (Figures 2A and 4B). We used it to immunoprecipitate RIN4 and test for association with RPM1, AvrB, or AvrRpm1 in plant extracts. We immunoprecipitated RIN4 from extracts of an *Arabidopsis* transgenic line expressing RPM1-myc from the native promoter (Boyes et al., 1998). We detected RPM1-myc specifically in the precipitated pellets when using the anti-RIN4 immune sera, but not when using the pre-immune sera from the same rabbit (Figure 1B). The relative amounts of protein from the immune pellet and the supernatant on the Western blot in Figure 1B are not equivalent; the pellet is over-represented by ten-fold. Thus, ~5%–10% of the RPM1-myc was coimmunoprecipitated by the anti-RIN4 sera. We next immunoprecipitated RIN4 from plants lacking RPM1 (*rpm1-3* has a stop codon at position 87 of *RPM1*; Grant et al., 1995) and transiently expressing functional AvrRpm1-HA or AvrB-HA under the control of a dexamethasone-inducible expression system (Aoyama and Chua, 1997; Nimchuk et al., 2000). Each Avr protein was coimmunoprecipitated by the immune, but not the preimmune, sera (Figure 1C). Approximately 1%–2% of AvrRpm1 or AvrB was in the pellet of the precipitations with the immune sera.

### RIN4 Is Required for the HR Induced by RPM1

*P. syringae* pathovar tomato (Pst) DC3000 carrying *avrRpm1* or *avrB* induces a robust, RPM1-dependent HR on *Arabidopsis* at 5 hr postinoculation (hpi; Dangl et al., 1992b). We determined that RIN4 is required for this HR. To reduce RIN4 levels, we generated transgenic lines of *Arabidopsis* (Col-0 background) that expressed antisense *RIN4* under the control of the strong 35S promoter from the cauliflower mosaic virus. In 55 of 88 primary transformants (T1s), RPM1-dependent HR was absent in at least one of three inoculated leaves (data not shown). In wild-type Col-0 plants, HR was observed on all 36 inoculated leaves. Many of these T1s had stunted morphology associated with strongly reduced RIN4 levels (data not shown, discussed below). A morphologically normal line with greatly reduced levels of RIN4 was carried to homozygosity (*rin4-as*). We measured levels of RIN4 in Col-0, in a Col-0 double mutant (*rpm1-3/rps2-101C*), and in *rin4-as* (Figure 2A). RPS2 is an *R* protein “closely” related to RPM1 that induces the HR and resistance against Pst DC3000 carrying *avrRpt2*. Using the *rin4-as* line, we determined the effect of reduced levels of RIN4 on the function of RPM1 and RPS2. First, we tested the ability of these plants to trigger the HR against isogenic strains of Pst DC3000 carrying either an empty plasmid (vector) or the same plasmid containing *avrRpm1*, *avrB*, or *avrRpt2*. Reduced levels of RIN4 severely compromised the HR induced by RPM1, but not that induced by RPS2 (Figure 2B).

We next used ion leakage, which correlates strongly with the HR (Baker et al., 1991), to quantify these differences (Figure 2C). In wild-type Col-0, ion leakage induced by Pst DC3000 carrying *avrRpm1*, *avrB*, and *avrRpt2* occurred earlier than that induced by Pst DC3000 carrying the vector. Likewise, Pst DC3000 (vector) induced more ion leakage than did buffer- or Pst DC3000/*hrcU*-inoculated controls. *HrcU* encodes a structural gene of the type III secretion system, and the mutant cannot deliver type III effectors into the host

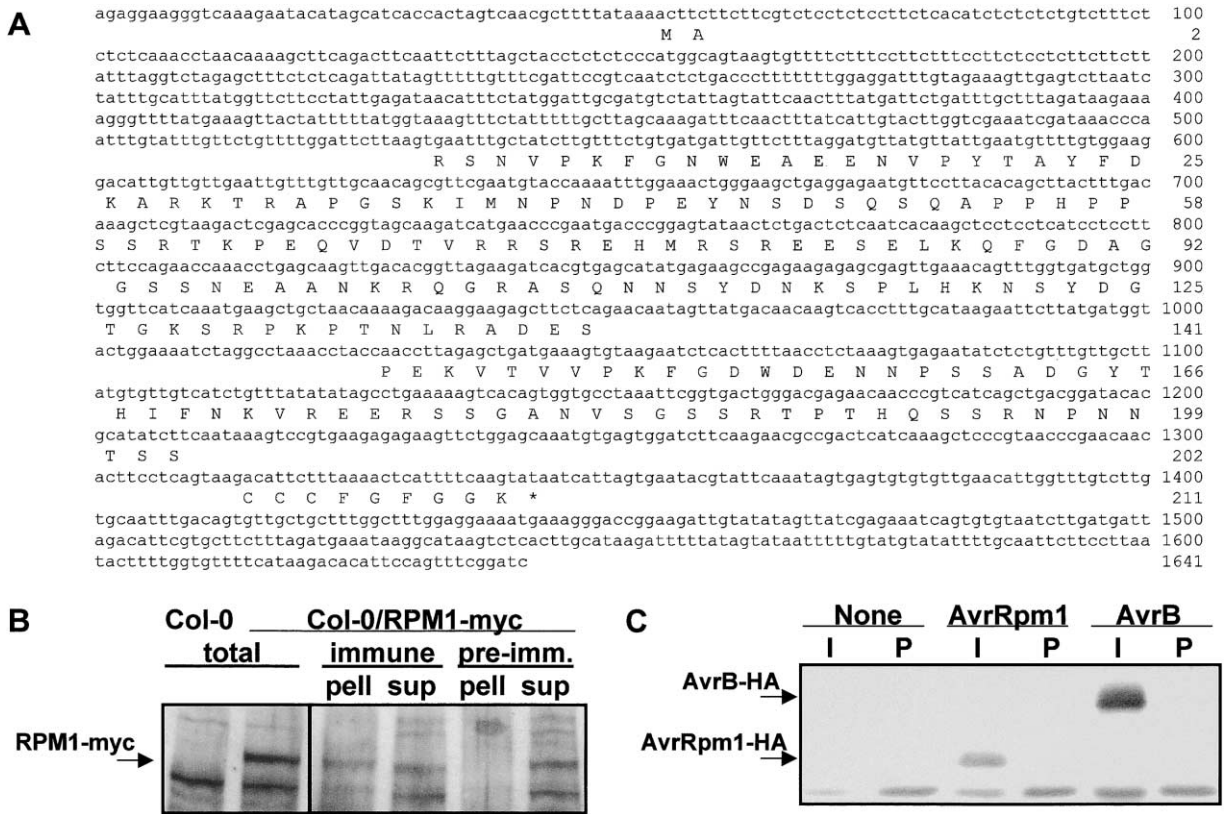


Figure 1. RIN4 Interacts with RPM1, AvrB, and AvrRpm1 in Plant Extracts

(A) Sequence from chromosome 3 of *Arabidopsis thaliana* containing *RIN4*. This portion of chromosome 3 is contained in P1 clone MJL12, and *RIN4* is annotated as GenBank gi15230250 and AGI gene number At3g25070. The DNA sequence of the 5'UTR, the coding sequence, and the 3'UTR is from cDNAs identified in the two-hybrid assay and by 5' RACE. The positions and DNA sequence of the introns is inferred by comparison with the genomic sequence of *Arabidopsis*.

(B) RPM1-myc coimmunoprecipitated with RIN4. Protein from Col-0/*rpm1-3*-expressing RPM1-myc under control of the native *RPM1* promoter was precipitated with anti-RIN4 antisera (immune) or sera from the same rabbit prior to exposure to the antigen (preimm.). Total extracts from Col-0 and Col-0/*RPM1-myc* as well as precipitated samples (pellet) and supernatants (sup) were subjected to an anti-myc Western blot.

(C) AvrB and AvrRpm1 coimmunoprecipitated with RIN4. Transgenic lines in *rpm1-3* that conditionally express AvrB-HA and AvrRpm1-HA were treated with dexamethasone (20  $\mu$ M). Leaf tissue was collected 60 hr later and proteins were immunoprecipitated with preimmune (P) or immune (I) sera against RIN4. Tissue from DEX-treated Col-0 (None) was included as a negative control. The precipitated samples were resolved on 12% SDS-PAGE and subjected to an anti-HA Western blot.

cell. Therefore, the ion leakage induced by Pst DC3000 (vector) is due to disease presumably caused by delivery of other type III effectors. The timing of increased ion leakage induced by Pst DC3000 expressing Avr proteins correlated with the timing of the HR induced by each (RPM1-dependent HR at 5 hpi; RPS2-dependent HR at 15 hpi). In *rpm1/rps2*, HR was absent and the disease-associated ion leakage induced by Pst DC3000 expressing Avr proteins was similar to that induced by Pst DC3000 (vector). In *rin4-as*, RPM1-mediated ion leakage was abrogated while that dependent on RPS2 was largely maintained. Thus, for both HR and the associated ion leakage, the requirement for RIN4 is specific to RPM1.

#### RIN4 Is Required for Accumulation of, and Cofractionates with, RPM1

We also suppressed the levels of RIN4 via antisense RNA in *RPM1-myc* transgenic plants. RIN4 levels in these plants correlated directly with RPM1-myc levels

(Figure 3A). In addition to the six lines shown in Figure 3, we observed the same correlation between levels of RIN4 and RPM1-myc in 35 additional T1 plants (data not shown). The levels of the *RPM1-myc* transcript were unaffected, even in lines with strongly suppressed levels of RIN4 and RPM1-myc (data not shown). We conclude that RIN4 is required for accumulation of RPM1 protein. Therefore, we asked whether RIN4 colocalizes with RPM1-myc. Like RPM1-myc (Boyes et al., 1998), RIN4 was localized to the cellular membrane fraction (Figure 3B). The accumulation and localization of RIN4 was unaffected in a mutant lacking RPM1 (*rpm1-3*). Thus, accumulation of RPM1-myc requires RIN4; accumulation of RIN4 and its localization to membranes does not require RPM1.

#### RIN4 Is Required for Normal Development of *Arabidopsis*

Reduction of RIN4 caused dwarfing in many plants. Plants expressing the lowest levels of RIN4 protein did

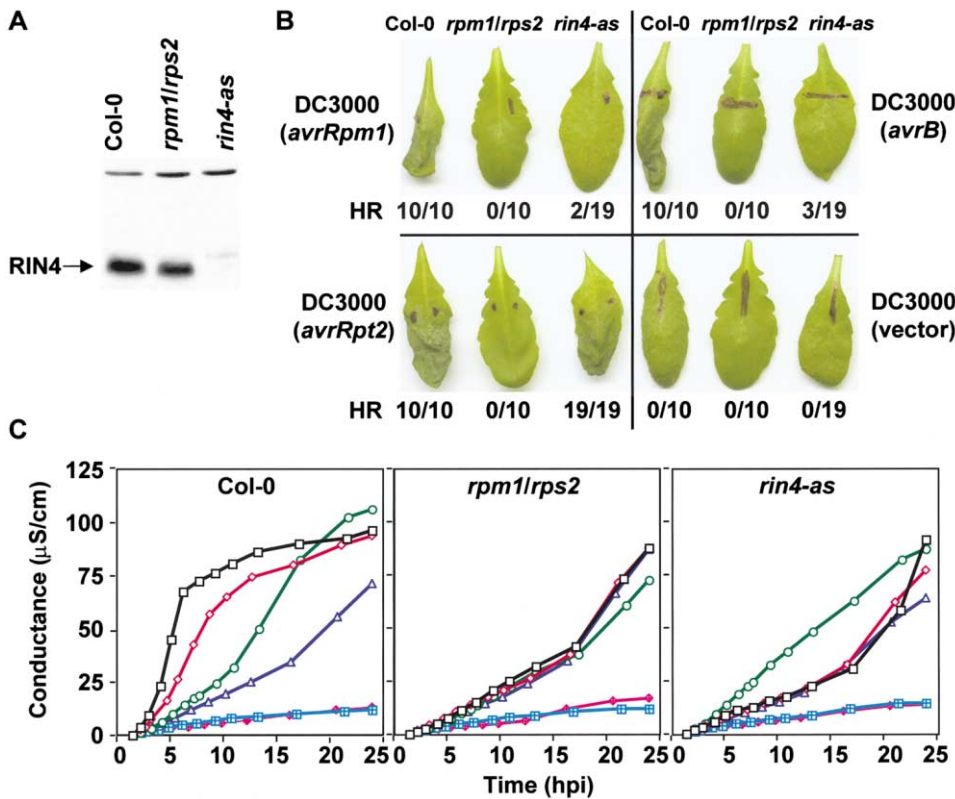


Figure 2. RIN4 Is Required for RPM1-Induced HR

(A) Western blot of RIN4 protein in Col-0, *rpm1/rps2*, and *rin4-as*.

(B) HR phenotypes of plant lines in (A) following inoculation with  $5 \times 10^7$  cfu/ml of *Pseudomonas syringae* pv. tomato (Pst) DC3000 carrying *avrRpm1*, *avrB*, *avrRpt2*, or empty vector. Representative leaves are shown 20 hr postinoculation. Beneath each pictured leaf is the number of leaves that showed a macroscopic HR from the total number of leaves infiltrated. Black marks are ink.

(C) Ion leakage measurements following inoculation with Pst DC3000 carrying *avrRpm1* (black squares), *avrB* (red diamonds), *avrRpt2* (green circles), empty vector (blue triangles), a mutation in *HrcU* (aqua hatched squares), or with buffer only (pink hatched diamonds). Following infiltration, 8 leaf discs per treatment were washed with H<sub>2</sub>O for 30 min then transferred to fresh H<sub>2</sub>O where conductance was measured ( $\mu\text{Siemens}/\text{cm}$ ) over time. The experiment is one example from three repetitions.

not progress beyond the seedling stage (data not shown). We therefore conclude that RIN4 is essential for viability of Col-0 seedlings. Also, RIN4 protein levels in many *rin4-as* lines varied from generation to generation, and even within a generation, presumably due to silencing of the anti sense construct (data not shown). We isolated a T-DNA insertion line in *Ws-0* (see Experimental Procedures) with an insertion at  $-35$  relative to the ATG of the *RIN4* open reading frame. This mutation (*rin4*) is recessive; *rin4* homozygotes were dwarfed relative to their wild-type and heterozygous siblings. The difference in size was first apparent at about 2.5 weeks of age and became more distinct as the plants grew (Figure 4A). Levels of RIN4 protein in *rin4* were greatly reduced relative to the levels in wild-type (Figure 4B). *rin4* plants formed dead cell lesions spontaneously late in their life-cycle, lacked apical dominance, and had poor fertility (data not shown). Growth in long day conditions exacerbated these phenotypes and additionally produced curled leaves. The altered morphology and protein levels in *rin4* were complemented by a transgene containing the 35S promoter driving *RIN4* with an amino-terminal T7 tag (Figures 4A and 4B). Complementation was observed in multiple lines (data not shown). A similar

suite of phenotypes was observed in the Col-0 anti sense lines. However, the lesions observed in Col-0 initiated along the central veins of the leaves while those in *Ws-0* initiated at the margins (data not shown). Also, the Col-0 anti sense lines did not lose apical dominance (data not shown).

#### RIN4 Is Required for RPM1-Dependent Inhibition of Bacterial Growth

The *rin4* insertion allele allowed detailed pathology experiments at developmental stages before obvious morphological changes began. We measured growth of Pst DC3000 (vector), (*avrRpm1*), (*avrB*), or (*avrRpt2*) on seedlings of *Ws-0*, *rin4*, and the complemented mutant (Figure 4C). Note that *Ws-0* encodes functional *RPM1* and *RPS2*. Plants were dip inoculated into bacterial suspensions and the number of bacteria per plant one hour ( $t = 0$ ), two days, and four days later was measured (see Experimental Procedures). Growth of Pst DC3000 (*avrRpm1*) or (*avrB*) on *rin4* was enhanced relative to that on *Ws-0* or the complemented mutant. RPM1 dependent growth inhibition was partially, but not fully, compromised in *rin4* (see below). Growth of Pst DC3000 (*avrRpt2*) was similar on all three plants. Consistent with

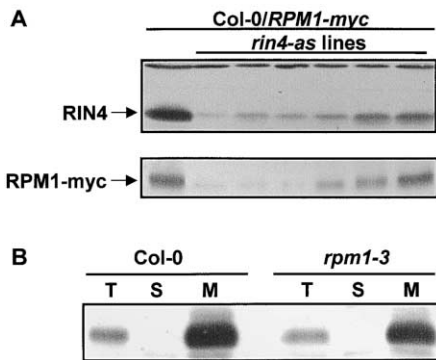


Figure 3. RIN4 Is Required for Accumulation of and Colocalizes with RPM1

(A) Total protein extracts were prepared from Col-0/RPM1-myc and six independent derivative lines expressing suppressed RIN4 levels. These extracts were subjected to anti-RIN4 (top) or anti-myc (bottom) Western blot.

(B) Col-0 and Col-0/rpm1-3 were crudely fractionated into soluble and membrane fractions. The total (T), soluble (S), and membrane (M) fractions were subjected to anti-RIN4 Western blot. The membrane fraction is comparatively over represented 5-fold by total protein yield.

the results for HR induction, RIN4 is therefore required for resistance mediated by RPM1, but not for resistance dependent on RPS2.

### RIN4 Negatively Regulates Basal Disease Resistance and *PR* Gene Expression

We observed that growth of Pst DC3000 (vector) on *rin4* plants was reduced significantly relative to that on both Ws-0 and the complemented *rin4* mutant (Figure 4C, left panel). Thus, *rin4* plants expressed heightened resistance against Pst DC3000. *rin4* plants also expressed heightened resistance to *Peronospora parasitica*, an oomycete pathogen (Figure 5). The Emco5 isolate of *P. parasitica* is virulent on Ws-0. The *Arabidopsis* accession La-er, Ws-0, *rin4*, and the complemented *rin4* mutant were spray inoculated with Emco5. Progression of infection after seven days was visualized with trypan blue (Figure 5A). Trypan blue stains pathogen structures and dead cells produced during the HR (as well as those produced in leaf veins during their development). La-er is resistant to Emco5 through the *RPP8* disease resistance gene (McDowell et al., 1998). Attempted infection on La-er gave rise to HR sites, virtually no hyphal growth was seen, and no sporangiophores were produced (data not shown). Emco5 grew robustly on Ws-0, producing hyphae throughout the leaf and many sporangiophores. Growth of Emco5 on *rin4* was greatly reduced relative to Ws-0. We observed limited hyphal growth and few sporangiophores. Instead, we observed numerous HR sites, reminiscent of those seen in La-er. Growth of Emco5 on the complemented mutant was similar to that on Ws-0. We used spore counts to quantify these visual

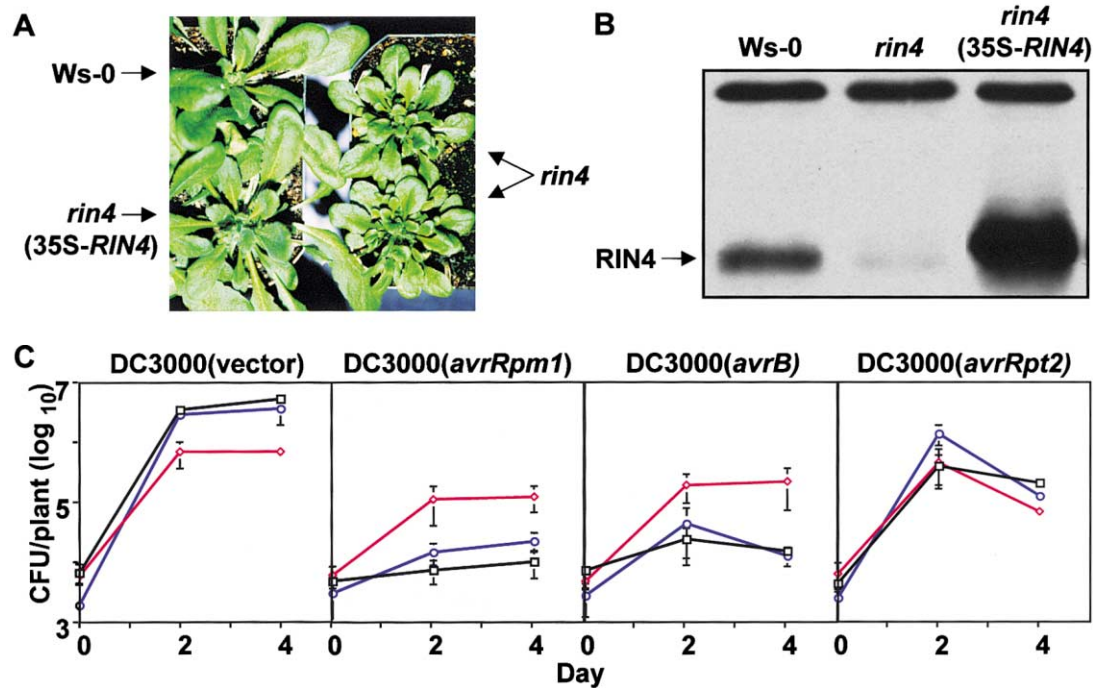


Figure 4. RIN4 Is Required for Inhibition of Bacterial Growth by RPM1

(A) A mutant (*rin4*) with a T-DNA insertion at -48 relative to the ATG of *RIN4* was identified in the ecotype Ws-0. Insertion of a 35S-T7 epitope tag-*RIN4* transgene (35S-*RIN4*) into *rin4* complemented this, and all other *rin4* phenotypes. The pictured plants were grown for 6 weeks in short days.

(B) Severely reduced RIN4 levels in the *rin4* insertion line, and overexpression in *rin4* (35S-*RIN4*). Total protein extracts were subjected to an anti-RIN4 Western blot. The larger size of RIN4 in the complemented mutant is due to the amino-terminal T7-tag.

(C) Growth of Pst DC3000-carrying vector, *avrRpm1*, *avrB*, or *avrRpt2* was measured on Ws-0 (black squares), *rin4* (red diamonds), and the complemented mutant (*rin4*/35S-*RIN4*-T7) (blue circles). Plants were 2 weeks old when infected.

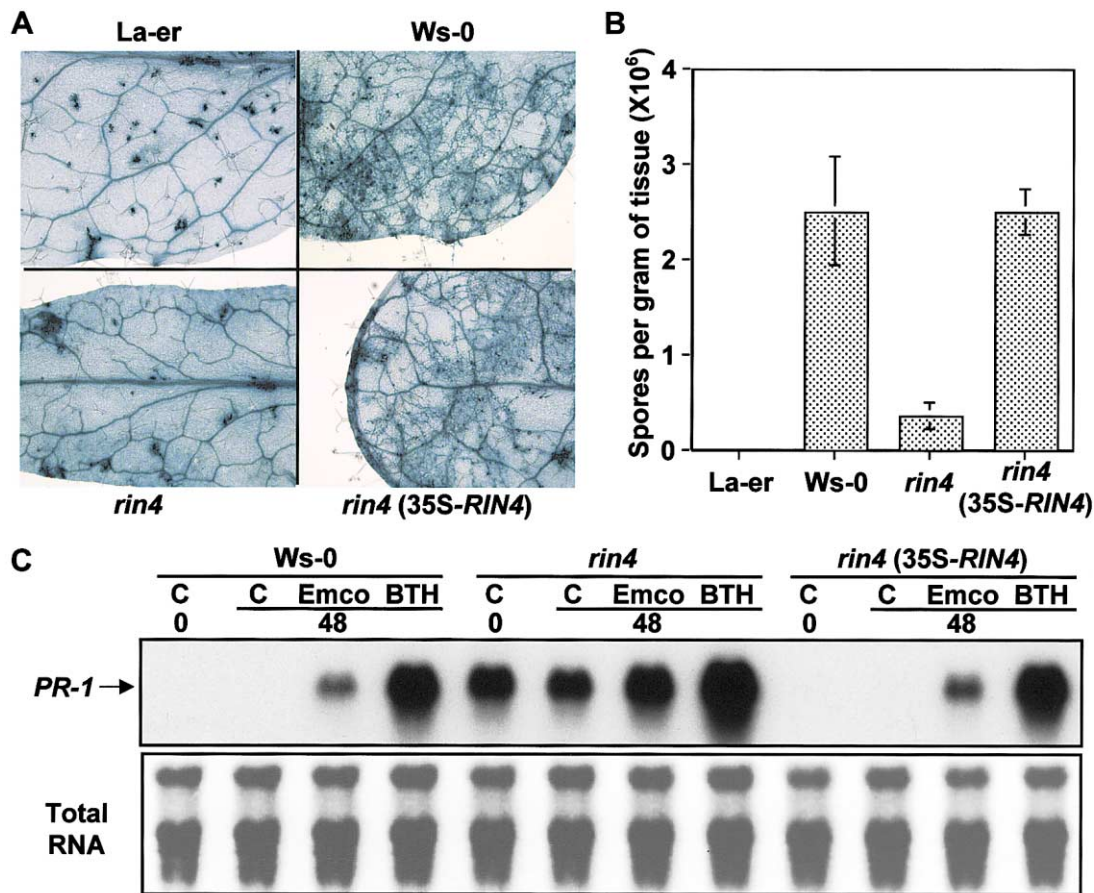


Figure 5. *RIN4* Is a Negative Regulator of Basal Defense and *PR-1* Transcription

(A) At 26 days of age, La-er, Ws-0, *rin4*, and *rin4* (35S-*RIN4*) were sprayed with 50,000 spores/ml of *Peronospora parasitica* isolate Emco5. Seven days after infection, leaves were trypan blue stained to visualize the progression of infection.

(B) Leaves from the infections in (A) were harvested at seven days after infection and the associated spores were counted and normalized to the weight of tissue.

(C) RNA blot analysis of *PR-1* expression. Plants from an experiment identical to that described in (A) were sprayed with a water control (C), 50,000 spores/ml of Emco 5, or BTH (0.35 mM). Samples were collected prior to and 48 hr after treatment. A negative image of ethidium bromide-stained total RNA is shown at bottom.

observations (Figure 5B). Infection of *rin4* plants produced ~15% as many spores as did infection of wild-type Ws-0 or the complemented mutant. No spore production occurred on La-er. We conclude that reduced levels of *RIN4* result in heightened resistance to two distinct, normally virulent pathogens, *P. syringae* and *P. parasitica*.

A common feature of *Arabidopsis* mutants expressing enhanced basal resistance to virulent pathogens is ectopic expression of pathogenesis-related (*PR*) genes. We therefore collected leaf tissue during a repetition of the experiment shown in Figures 5A and 5B for RNA analysis. We collected tissue at the time of, and 48 hr after, treatment with water, *P. parasitica* Emco5, or the salicylic acid analog benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH; 0.35 mM). We probed the RNA blot with *PR-1*, a common marker of systemic acquired resistance and basal defense responses (Figure 5C). In Ws-0 leaves, Emco5 and BTH treatment induced *PR-1* transcription as expected. We also observed induction of *PR-1* expression in the complemented *rin4* mutant. However, in *rin4*, *PR-1* was constitutively ex-

pressed. Interestingly, treatment of *rin4* with Emco5 and BTH resulted in more total *PR-1* mRNA than in the wild-type or the complemented mutant. *rin4* plants also constitutively expressed *PR-5* (data not shown). Therefore, like other mutants in *Arabidopsis* with enhanced basal disease resistance, *rin4* constitutively expresses *PR* genes.

#### AvrRpm1 or AvrB Induces RPM1-Independent Phosphorylation of *RIN4*

AvrRpm1 and AvrB induced a posttranslational modification of *RIN4*. We first observed this when the type III effectors AvrRpm1 and AvrB were delivered by inoculation with Pst DC3000 (Figure 6A). *RIN4* was modified in a way that reduced its mobility in SDS-PAGE. The mobility shift induced by AvrRpm1 was detectable by 2 hpi, and that induced by AvrB was detectable by 8 hpi. The modification of *RIN4* occurred in *rpm1-3* plants that lack RPM1 (Figure 6A, lower panel). The modification was also induced when the type III effectors are expressed in the absence of *P. syringae* (Figure 6B). For this experiment, we used *rpm1-3* plants containing transgenes confer-

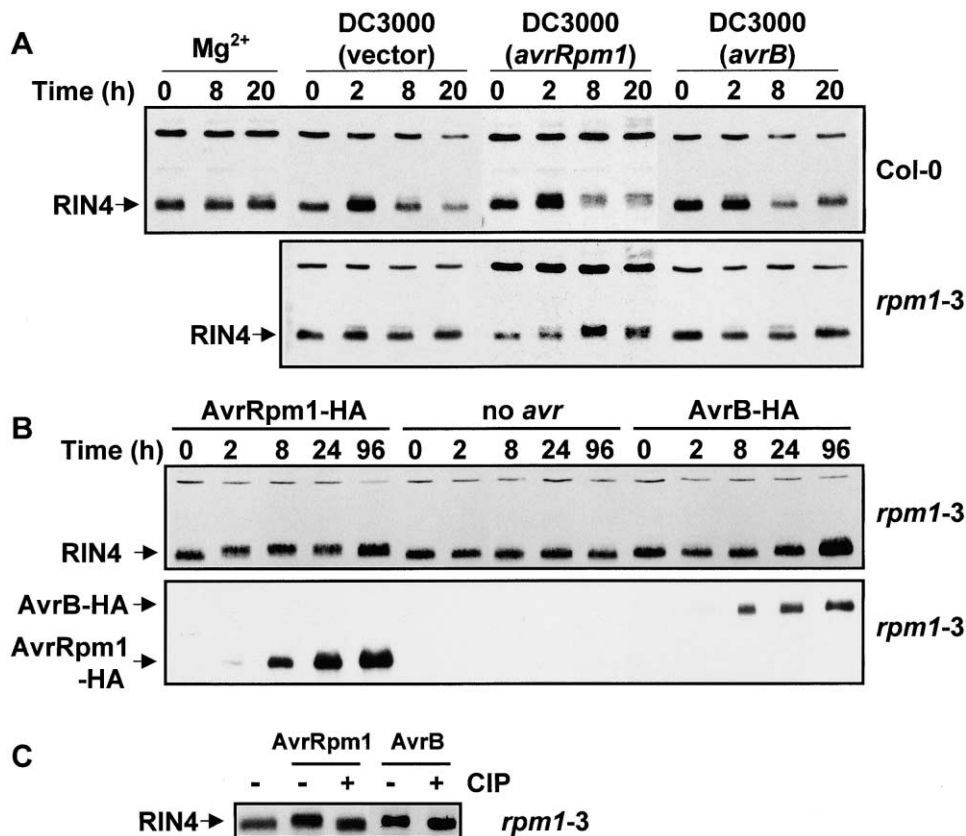


Figure 6. AvrRpm1 and AvrB Induce Phosphorylation of RIN4

(A) Col-0 (upper panel) and *rpm1-3* (lower panel) plants were infiltrated with buffer or  $5 \times 10^7$  cfu/ml Pst DC3000-carrying empty vector, *avrRpm1*, or *avrB*. Samples were collected over time and total protein extracts were subjected to an anti-RIN4 Western blot. (B) Transgenic *rpm1-3* lines expressing dexamethasone inducible AvrRpm1-HA or AvrB-HA were sprayed with DEX (20  $\mu$ M). Samples were collected over time and total protein extracts were subjected to an anti-RIN4 Western (upper panel) and an anti-HA Western (lower panel). (C) Samples from leaves expressing no Avr protein, AvrRpm1, or AvrB (24 hr samples from panel B) were either mock treated (-) or treated (+) with calf alkaline intestinal phosphatase (CIP) and subjected to anti-RIN4 Western.

ring dexamethasone-inducible expression of AvrRpm1-HA or AvrB-HA (Aoyama and Chua, 1997; see Experimental Procedures). AvrRpm1 and AvrB expression was induced by spraying plants with dexamethasone and their accumulation was monitored by anti-HA Western blot (Figure 6B, lower panel). Conditional expression of AvrRpm1 rapidly induced the mobility shift of RIN4 (Figure 6B, upper panel). The mobility shift induced by AvrB was observed prior to treatment with dexamethasone and is attributed to "leaky" expression of AvrB. We also noted an increase in RIN4 levels 96 hr after induction of AvrB. This increase was mirrored by *RIN4* mRNA accumulation in similar experiments (Z. Nimchuk and J.L.D., unpublished). Therefore, AvrRpm1 and AvrB induce modification of RIN4 independent of *P. syringae* and independent of RPM1. Calf alkaline intestinal phosphatase restored the mobility of modified RIN4 to that of the unmodified form (Figure 6C), indicating that the slowly migrating form had been phosphorylated.

#### Discussion

We demonstrate that RIN4 interacts with AvrRpm1, AvrB, and RPM1. Accumulation and function of RPM1 requires RIN4. RIN4 can negatively regulate basal de-

fense responses. Finally, we show that AvrRpm1 or AvrB induces phosphorylation of RIN4. We propose a model in which RIN4 is a target of the virulence activities of AvrRpm1 and AvrB (Figure 7). AvrRpm1 is, in fact, required for full virulence in several *P. syringae* pv. *maculicola* strains (Ritter and Dangl, 1995; L. Rohmer and J.L.D., in preparation), and AvrB can add to the virulence of a *P. syringae* strain pathogenic on soybean (Ashfield et al., 1995). We hypothesize that the interaction with and/or the phosphorylation of RIN4 by AvrRpm1 and AvrB underlies their virulence activities. If the interaction/phosphorylation enhances the activity of RIN4 as a negative regulator of basal plant defense, then the virulence of *P. syringae* carrying either of these type III effectors could be enhanced (so long as those plants lack *RPM1*). The model also predicts that RPM1 "guards" the plant against pathogens that manipulate RIN4 (Dangl and Jones, 2001).

Do RPM1 and RIN4 stably associate in the plant? RIN4 is required for accumulation of RPM1. However, only a small percentage of RPM1 protein is coimmunoprecipitated with RIN4. The association of RPM1 and RIN4 may be (1) a transient association required for subsequent stability of RPM1, (2) stable in the plant but not stable through the course of the co-IP protocol, or (3) saturated

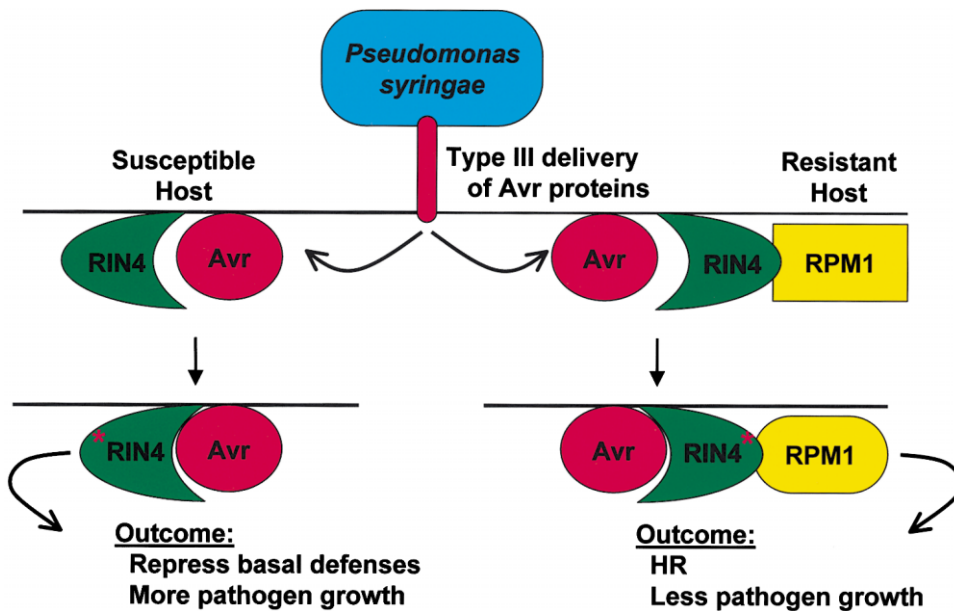


Figure 7. Model: RIN4 Is a Virulence Target for Type III Effectors that Is Guarded by RPM1

AvrRpm1 or AvrB (Avr) are delivered into the plant cell via type III secretion. In susceptible *rpm1* plants, the two type III effectors can associate with and induce phosphorylation of RIN4 (\*). This modification is speculated to enhance or "lock on" RIN4 activity as a negative regulator of basal defenses. In resistant *RPM1* plants, RIN4 associates with RPM1 and is required for its accumulation. The two type III effectors associate with and induce phosphorylation of RIN4, as in the susceptible host. In the resistant host, however, this interaction and/or modification activates RPM1-mediated disease resistance, including the HR.

by an excess of RPM1. RIN4, like RPM1, exists in the membrane fraction of the plant cell. Steady-state colocalization of RPM1 and RIN4 is consistent with a model in which the two proteins stably interact. While RIN4 is required for accumulation of RPM1, the converse is not true. Levels of RIN4 are unaffected in *rpm1-3*, where mutation introduces a stop codon at amino acid 87, and in ecotypes of *Arabidopsis* that lack *RPM1* (data not shown). RPM1 also is not required for localization of RIN4 to a membrane fraction. Thus, RIN4 is required for accumulation of RPM1 at the membrane by either stably interacting with it or by otherwise mediating its stabilization.

An interesting question that arises from our studies is whether RIN4 phosphorylation affects its binding to RPM1. However, the fact that the RPM1 protein disappears with the onset of the HR (Boyes et al., 1998) limits our capacity to address this question. Any sample in which the majority of RIN4 is phosphorylated no longer contains detectable levels of RPM1. Similarly, we are unable to test whether RPM1, RIN4, and AvrRpm1 or AvrB associate in a single complex.

RIN4 functions as a negative regulator of basal defense responses, since the *rin4* mutant displays heightened resistance against two normally virulent pathogens. The *rin4* mutant constitutively expresses *PR-1* and *PR-5*. The enhanced basal defense of *rin4* is likely to result from the constitutive expression of these and other defense genes. The complemented *rin4* mutant overexpresses RIN4. Overexpression of a negative regulator of basal defenses could result in plants with heightened susceptibility to pathogens in general. This does not appear to be the case for avirulent pathogens. On

our *RIN4* overexpressing plants, resistance specified by *RPM1* was not compromised. We also did not observe heightened susceptibility to virulent pathogens in these plants. The pathogen isolates used (*Pst* DC3000 and *P. parasitica* Emco5) are highly virulent, therefore, enhanced growth could be difficult to detect. Tests with less aggressive, virulent pathogens may reveal that overexpression of RIN4 does enhance suppression of basal defense. Alternatively, the wild-type levels of RIN4 could be saturating for its negative regulatory function, and thus, no additional susceptibility would be achieved in *RIN4*-overexpressing lines.

Numerous *Arabidopsis* mutants induce constitutive defenses in plants (Beers and McDowell, 2001; Dangl et al., 1996). Similar to *rin4*, many of these mutants are dwarfed and form cell death lesions spontaneously. Most were identified in screens for plants with heightened resistance against virulent pathogens, screens for plants that spontaneously form lesions, or screens for plants that constitutively express *PR* genes. Because RIN4 is essential for *Arabidopsis* viability, it was logically not identified in these screens. For the same reason, RIN4 was probably not isolated in a large screen for loss of RPM1 activity (Tornero et al., 2002). It is unclear whether the genes defined by these constitutive defense mutations have wild-type functions in either specific or basal plant disease resistance, or whether their mutant phenotypes reflect perturbation of cellular homeostasis that leads inexorably to ectopic cell death and activation of basal defense. The specific requirement of RIN4 for RPM1 function, however, in addition to its deduced role as a negative regulator of basal defense, supports our conclusion that the *rin4* phenotype is likely to reflect its

wild-type function. The *rin4* phenotype is, in any case, unique among this mutant class.

There is precedence for pathogen effectors inhibiting plant defense. Mutation in *Arabidopsis* *PMR1* (powdery mildew resistant 1) leads to diminished growth of the powdery mildew fungal pathogen (Vogel and Somerville, 2000). Vogel and Somerville hypothesized that an effector(s) from powdery mildew targets *PMR1* to suppress basal defense. Unlike plants lacking *RIN4*, however, plants lacking *PMR1* do not activate constitutive defenses. Despite these differences, *PMR1* and *RIN4* may be similarly targeted by pathogens in order to suppress host defenses. *P. syringae* type III effectors can inhibit *R* function in response to another type III effector. For example, *AvrRpt2* can block the ability of *Arabidopsis* to respond to *AvrRpm1* (Ritter and Dangl, 1995). Similarly, *AvrPphC* can block the ability of bean to respond to *AvrPphF* (Tsiamis et al., 2000). Our data suggest that *AvrRpm1* and *AvrB* suppress basal defenses through manipulation of *RIN4*. Similarly, *AvrRpt2* expression can delay the onset of defense gene activation in some *rps2 Arabidopsis* accessions (Chen et al., 2000). Effectors evolved to repress defenses of the plant are potentially widespread among plant pathogens.

*AvrRpm1* and *AvrB* target *RIN4* for phosphorylation. We hypothesize that the interaction of these *Avr* proteins with *RIN4* is required for the induced modification. However, while the *RIN4* phosphorylation induced by these two *Avr* proteins is quantitative, only a small percentage of *AvrRpm1* or *AvrB* coimmunoprecipitated with *RIN4*. The most likely explanation for this is that the plant tissue used contained an excess of *AvrRpm1* and *AvrB*. In the dexamethasone-inducible transgenic lines, phosphorylation of *RIN4* occurred prior to, and 2 hr after, induction of *AvrB* and *AvrRpm1*, respectively. At these times, levels of the *Avr* proteins were barely detectable or undetectable. The co-IP samples were collected 60 hr after induction. At this time, the *Avr* proteins are expressed at a much higher level than that demonstrably sufficient to modify *RIN4*. Thus, the fraction of *AvrRpm1* and *AvrB* sufficient to induce phosphorylation of *RIN4* may constitute the same fraction associated with *RIN4*.

Manipulation of *RIN4* may enhance the basal defenses of the plant. The interaction with, and phosphorylation of, *RIN4* mediated by *AvrRpm1* and *AvrB* occurs independently of *RPM1*. In the absence of *RPM1*, *AvrB* also induces increased levels of *RIN4* protein by increasing transcription of *RIN4* (Z. Nimchuk and J.L.D., unpublished). The phosphorylation of *RIN4* (or increase in *RIN4* levels) induced by these type III effectors may enhance its activity as a negative regulator of basal defenses of the plant. It is unsurprising that phosphorylation status changes following infection (Dietrich et al., 1990). Indeed, pharmacological studies demonstrate a requirement for phosphorylation in defense responses and various kinases are activated following the induction of *R*-gene-specified resistance (reviewed in Scheel, 1998). These examples correlate phosphorylation with activation and are at first glance contrary to our proposal, that phosphorylation of *RIN4* causes suppression of basal defenses. However, MAP kinase 4 of *Arabidopsis* can negatively regulate systemic acquired resistance (Petersen et al., 2000). We thus propose that phosphoryla-

tion of *RIN4* by *AvrRpm1* and *AvrB* enhances its activity as a repressor of basal defenses.

We also propose that *RPM1* activation is dependent on the manipulation of *RIN4* by *AvrRpm1* or *AvrB*. It is tempting to speculate that phosphorylation of *RIN4* contributes to this activation. The timing of *RIN4* phosphorylation induced by *AvrB* delivered from *P. syringae* (between 2 and 8 hr) is slower than that induced by *AvrRpm1* (by 2 hr). This timing mirrors the slower increase of cytosolic calcium induced by *AvrB* (Grant et al., 2000). Calcium influx is necessary for induction of the HR by *RPM1* (Grant et al., 2000). Also, ion leakage induced by *Pst* DC3000 (*avrB*) lags behind that induced by *Pst* DC3000 (*avrRpm1*). The slower activity of *AvrB* in each of these read-outs is consistent with the hypothesis that phosphorylation of *RIN4* is necessary for the induction of the *RPM1*-dependent HR. *RPM1* could be recruited to a complex containing *Avr* protein and phosphorylated *RIN4*, and defense responses and HR thus activated. Alternatively, *RPM1* could be displaced from a complex containing *RIN4* upon recruitment of either *AvrRpm1* or *AvrB* to that complex. Displacement of *RPM1* would then result in its activation. This model is reminiscent of the oligomerization and activation of *Apaf-1*, with which the plant NB-LRR proteins share structural similarity in the NB-ARC domain (reviewed in Budihardjo et al., 1999; Aravind et al., 1999; van der Biezen and Jones, 1998a). The *Pto* kinase of tomato interacts with and specifies resistance to *AvrPto* from *P. syringae*. *Prf* (an NB-LRR class protein) is required for function of, and is genetically downstream of *Pto* (reviewed in Ellis, 2000; van der Biezen and Jones, 1998b). *RIN4* and *Pto* may serve similar functions as protein targets of type III effectors, and by extension, the elicitors of multiple *R* proteins may arise from pathogen-induced phosphorylation of plant proteins. Thus, *RPM1* may “guard” the plant against pathogens that induce phosphorylation of *RIN4*, and other NB-LRR proteins may guard additional cellular targets against a spectrum of virulence factors (Dangl and Jones, 2001).

A domain of *R* proteins that contributes to their specificity may lie outside of the LRRs. Specificity for *R* proteins has been genetically ascribed to the LRR domain, but only the Pi-ta interaction with *AvrPi-ta* (Jia et al., 2000) provides evidence for binding of an LRR domain to an *Avr*-dependent signal. The sequences of *AvrRpm1* and *AvrB* are unrelated yet they both interact with, and induce phosphorylation of, *RIN4*. The region of *RPM1* that interacts with *RIN4* comprises another “variable domain” among the NB-LRR class of *R* proteins. Between the amino-terminal CC (or TIR) domain, and the amino-terminal end of the NB domain, is a stretch of roughly 150 amino acids that is highly variable. It may be that this domain is a common interaction platform for a variety of cellular proteins that are targets of virulence factors. In support of this notion, the amino acids that are highly variable, and can underlie specificity, among *L* alleles reside in both the TIR and the domain just carboxyl to it. These amino acid positions, like the LRRs, are under variable levels of diversifying selection (Luck et al., 2000), and these domains together may specify interaction with the “guardee.” Thus, variability in this N-terminal domain of *R* genes may reflect the diversity of guardee host targets.

It is by now well established that bacterial Avr proteins can function as virulence factors inside the cells of the plant (reviewed in Nimchuk et al., 2001). Collectively, these data suggest that virulence activity-induced perturbation of plant homeostasis by type III effector proteins may initiate R function. R proteins might therefore function as receptors recognizing biochemical signatures wrought from the virulence functions of Avr proteins. Thus, for an Avr protein to evade recognition by the corresponding R protein, it may have to discard the activity that allows it to function as a virulence factor. It is interesting to note that many *P. syringae avr* genes (including *avrRpm1* and *avrB*) are not widespread among different closely related pathogenic isolates. This suggests the existence of a class of type III effectors that cannot mutate (without loss of virulence) to avoid detection, and hence are replaced wholesale on an evolutionary timescale. In this case, sequence-unrelated type III effectors may evolve independently to target the same host proteins, as demonstrated here for AvrRpm1 and AvrB. Furthermore, sequence unrelated type III effectors that target the same host proteins could be recognized by the same R protein, as is also the case for AvrRpm1 and AvrB. A corollary of this notion is that a single host target of virulence function could be guarded by more than one R protein (Dangl and Jones, 2001). Our identification of RIN4 as a common target of AvrRpm1 and AvrB begins to unravel the means by which RPM1 expresses dual specificity. Our results further suggest that the diversity of type III effector and R protein interactions leading to disease resistance may account for the diversity in host targets of type III effector virulence functions.

## Experimental Procedures

### DNA

Baits for the yeast two-hybrid assay were cloned into pEG202. The library screened in yeast was in pUG4-5 (Gyurius et al., 1996). For inducible expression of AvrRpm1 and AvrB in planta, each gene with a carboxy-terminal glycine and HA tag was cloned into pTA7002 (Aoyama and Chua, 1997). For production of RIN4 in *E. coli*, the carboxy-terminal ~2/3 of *RIN4* (encoding amino acids 77-211) was cloned into pGEX-6P (Amersham Pharmacia). For expression of RIN4 in planta, the entire cDNA with an amino-terminal T7 tag was cloned in the sense orientation into pBAR-35S (B.F.H., unpublished). For antisense suppression of *RIN4*, the entire cDNA and 153 base pairs of 5' UTR were cloned in the antisense orientation into pBAR-35S.

### Protein

GST-RIN4 (amino acids 77-211) was produced in BL21 (pLysS) cells (Novagen) and purified with glutathione Sepharose CL-4B (Amersham Pharmacia). RIN4 was separated from GST by cleavage with PreScission Protease (Amersham Pharmacia). Polyclonal antisera was raised in rabbits (Covance Research Products).

Total protein extracts were prepared by grinding 1 g of tissue per 5 ml 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  $20,000 \times g$  for 10 min at 4°C. Concentration of protein in the supernatant was determined with the Bio-Rad protein assay (Bio-Rad). Samples were separated on SDS-PAGE gels (mini protean, Bio-Rad) of 7.5% for RPM1-myc or 12% for RIN4, AvrRpm1-HA, and AvrB-HA and transferred to nitrocellulose. Western blots were done by standard methods. Anti-RIN4 sera was used at 1:2000. Detection of RPM1-myc was with the mouse monoclonal antibody 9E10.

For immunoprecipitations, tissue was first ground in liquid nitro-

gen with a mortar and pestle. This material was then homogenized (Polytron, Kinematica) in 2 ml of buffer (50 mM HEPES [pH 7.5], 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  $20,000 \times g$  for 20 min at 4°C. Four hundred  $\mu$ l of this supernatant was combined with 5  $\mu$ l of preimmune or anti-RIN4 sera. After incubation at 4°C for 1 hr, 25  $\mu$ l of protein A-agarose (Boehringer Mannheim) was added and the reaction was rolled at 4°C for 6 hr. The resin was washed 4 times in 1 ml of the same buffer (except 0.1% Triton X-100 instead of 0.2%).

Membrane proteins were fractionated by grinding 1 g of tissue per 10 ml of buffer (10 mM Tris-HCl [pH 7.0], 0.33 M sucrose, 1 mM EDTA, and plant protease inhibitor cocktail [Sigma-Aldrich]) and pelleting insoluble debris by centrifugation at  $20,000 \times g$  for 20 min at 4°C. The supernatant of this spin was the total (T) fraction. Ten  $\mu$ l of 1 M CaCl<sub>2</sub> was added to 500  $\mu$ l of the total fraction and membranes were pelleted at  $50,000 \times g$  for 90 min at 4°C. The supernatant of this spin was the soluble (S) fraction and the pellet was resuspended in 100  $\mu$ l of buffer to form the membrane (M) fraction.

Proteins were tested for phosphorylation by treatment with calf alkaline intestinal phosphatase (CIP). An extract was prepared by grinding 1 g of tissue per 5 ml of buffer (50 mM Tris-HCl [pH 8.0], 1% Triton X-100, 1 mM EDTA, and plant protease inhibitor cocktail [Sigma-Aldrich]). Insoluble material was pelleted by two rounds of centrifugation at  $20,000 \times g$  for 10 min at 4°C. The concentration of protein in the supernatant was determined with the Bio-Rad protein assay. The supernatant was then diluted ~1:15 in a reaction containing  $1 \times$  buffer #3 (New England Biolabs) and containing or lacking 10 units of CIP per 25  $\mu$ g of total protein (New England Biolabs). Following incubation at 37°C for 90 min, SDS-sample buffer was added directly to the reaction.

### Yeast

All experiments used the yeast strain EGY48 (Finley and Brent, 1996). Transformations used the Frozen-EZ Yeast Transformation II Kit (Zymo Research). The library used in the yeast two-hybrid assay contained cDNAs from *Arabidopsis* (Col-0). The tissue came from both unchallenged plants and plants challenged 1 to 3 hr prior with DC3000-expressing AvrRpm1. Baits were confirmed to be in the nucleus by virtue of their ability to repress lacZ transcription from pJK101 (Finley and Brent, 1996).

### Transgenic Plants

The T-DNA insertion allele *rin4* was isolated from the *Arabidopsis* Functional Genomics Consortium (AFGC) lines by PCR screening with a primer in the T-DNA border pointing outward and a primer spanning the stop codon of *RIN4* pointing toward the start codon (<http://www.biotech.wisc.edu/Arabidopsis/>).

Transgenic plants were generated by vacuum infiltrating *Agrobacterium tumefaciens* (GV3101) into flowering *Arabidopsis* (Clough and Bent, 1998). Transgenic progeny were selected by spraying with BASTA (AgrEvo) (for pBAR-35S) of growth on plates of Gamborg's B5 (Gibco) with 20  $\mu$ M hygromycin B (Sigma) (for pTA7002). Lines with single insertion loci were identified and carried to homozygosity.

Plants expressing RPM1-myc have been previously described (Boyes et al., 1998). The line used in these experiments contains the same transgene in the *rpm1-3* background (Col-0/*rpm1-3/RPM1-myc*). Briefly, these plants are homozygous for a transgene containing the native *RPM1* promoter upstream of the *RPM1* gene that has a carboxy-terminal tag of 4.5 copies of the myc epitope.

Plants that inducibly express AvrRpm1 and AvrB were generated in *rpm1-3* (an allele of *RPM1* with a stop codon at amino acid 87; Grant et al., 1995). For the experiments in Figures 1C and 6B, 3-week-old plants were sprayed with 20  $\mu$ M dexamethasone (Sigma) and 0.0075% silwet L-77 (CKWitco Corporation). For the experiment in Figure 1C, tissue was harvested 60 hr later.

### *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 RIN4 mobility shift assays, DC3000 was resuspended at 50 million CFU/ml in 10

mM MgCl<sub>2</sub>. Bacteria were infiltrated into leaves of 4- to 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 monitored principally 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<sub>2</sub> and 0.02% Silwet L-77 and kept covered for 24 hr. At one hour after dipping ( $t = 0$ ), day 2, and day 4, the number of CFUs per plant was determined. Four plants (whole aerial tissue) were placed in 1 ml of 10 mM MgCl<sub>2</sub> 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.

#### *Peronospora parasitica*

Emc5 was propagated on the *Arabidopsis* ecotype Ws-0 (Dangl et al., 1992a). Spores (50,000/ml in water) were spray inoculated onto 26-day-old plants and the plants were subsequently maintained in 100% humidity. Leaves were trypan blue stained by heating to 95°C for 5 min in the presence of excess dye solution (70% ethanol, 7% phenol, 7% lactic acid, 7% glycerin, and 0.07 mg/ml trypan blue [Sigma]). After staining, leaves were washed for 3 days in chloral hydrate (2.5 mg/ml in water) with several changes of wash solution. Spore counts were conducted by harvesting tissue 7 days after infection. Tissue was weighed and then spores were collected by vortexing tissue in water. Spores were counted in a hemocytometer (Hausser Scientific).

#### RNA Analyses

To prepare RNA, leaves were collected prior to or 48 hr after the indicated treatments and ground by mortar and pestle in liquid nitrogen. RNA was extracted with Trizol following the manufacturers 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 as per manufacturer's directions. The probe for the *PR-1* northern was generated by pcr and labeled with  $\alpha$ -ATP using reagents provided in the Prime-It II random primer labeling kit (Stratagene). cDNAs containing the entire RIN4 ORF were isolated using the SMART Race cDNA amplification kit (Clontech).

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