Arabidopsis RIN4 Negatively Regulates Disease Resistance Mediated by RPS2 and RPM1 Downstream or Independent of the NDR1 Signal Modulator and Is Not Required for the Virulence Functions of Bacterial Type III Effectors AvrRpt2 or AvrRpm1

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Bacterial pathogens deliver type III effector proteins into the plant cell during infection. On susceptible (r) hosts, type III effectors can contribute to virulence. Some trigger the action of specific disease resistance (R) gene products. The activation of R proteins can occur indirectly via modification of a host target. Thus, at least some type III effectors are recognized at site(s) where they may act as virulence factors. These data indicate that a type III effector’s host target might be required for both initiation of R function in resistant plants and pathogen virulence in susceptible plants. In Arabidopsis thaliana, RPM1-interacting protein 4 (RIN4) associates with both the Resistance to Pseudomonas syringae pv maculicola 1 (RPM1) and Resistance to P. syringae 2 (RPS2) disease resistance proteins. RIN4 is posttranslationally modified after delivery of the P. syringae type III effectors AvrRpm1, AvrB, or AvrRpt2 to plant cells. Thus, RIN4 may be a target for virulence functions of these type III effectors. We demonstrate that RIN4 is not the only host target for AvrRpm1 and AvrRpt2 in susceptible plants because its elimination does not diminish their virulence functions. In fact, RIN4 negatively regulates AvrRpt2 virulence function. RIN4 also negatively regulates inappropriate activation of both RPM1 and RPS2. Inappropriate activation of RPS2 is nonspecific disease resistance 1 (NDR1) independent, in contrast with the established requirement for NDR1 during AvrRpt2-dependent RPS2 activation. Thus, RIN4 acts either cooperatively, downstream, or independently of NDR1 to negatively regulate RPS2 in the absence of pathogen. We propose that many P. syringae type III effectors have more than one target in the host cell. We suggest that a limited set of these targets, perhaps only one, are associated with R proteins. Thus, whereas any pathogen virulence factor may have multiple targets, the perturbation of only one is necessary and sufficient for R activation.

INTRODUCTION

In response to the pressures of infection, plants evolved an immune system to specifically detect pathogens and induce defenses against them. The most efficient sentinels of the plant immune response are proteins encoded by the disease resistance (R) genes (Flor, 1971). The most common and widely distributed class of R proteins has a central nucleotide binding site (NB) domain and C-terminal Leu-rich repeats (LRRs). Some of these so-called NB-LRR R proteins have N termini with homology to the intercellular portion of the Drosophila Toll and mammalian interleukin (IL-1) receptors (TIR-NB-LRR). Other R proteins have a coiled-coil (CC) motif at their N termini (CC-NB-LRR) (Dangl and Jones, 2001). Activation of NB-LRR proteins induces a defense response consisting of a series of biochemical and cellular events and massive transcriptional reprogramming within and surrounding the infection site (McDowell and Dangl, 2000; Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003; Nimchuk et al., 2003). These often, but not always, culminate in a localized programmed cell death called the hypersensitive response (HR).

Plant pathogenic bacteria express genes whose products trigger activation of specific NB-LRR R proteins. These were historically termed avr genes because their presence rendered strains expressing them avirulent on plants expressing the corresponding R gene (Staskawicz et al., 1984). These Avr proteins are substrates of the evolutionarily conserved type III secretion system used by a variety of Gram-negative animal and plant pathogens to deliver type III effector proteins to the eukaryotic host cell (Staskawicz et al., 2001; Colmer et al., 2002; Greenberg and Vinatzer, 2003). Thus, type III effector proteins in general, including the operationally defined Avr...
proteins, are likely to function primarily as virulence factors contributing to pathogen fitness on susceptible hosts. A growing base of experimental evidence supports this notion (Keamey and Staskawicz, 1990; Lorang et al., 1994; Ritter and Dangl, 1995; Chang et al., 2000; Chen et al., 2000; reviewed in Nimchuk et al., 2001).

The simplest molecular explanation for the genetics of avr-R disease resistance systems postulated a direct ligand–receptor interaction, but there is little experimental evidence to generally support this model with respect to NB-LRR proteins. This paucity of data led to the articulation of an alternative hypothesis in which R proteins monitor the integrity of host targets of pathogen virulence factors (Van der Biezen and Jones, 1998; Dangl and Jones, 2001; Van der Hoorn et al., 2002; Mackey, 2004). Experimental support for this guard hypothesis is mounting (Kruger et al., 2002; Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003; Shao et al., 2003).

Resistance to Pseudomonas syringae pv maculicola 1 (RPM1) encodes a CC-NB-LRR R protein that confers resistance against P. syringae expressing either of two sequence unrelated type III effectors, AvrB and AvrRpm1 (Bisgrove et al., 1994; Grant et al., 1995). RPM1-interacting protein 4 (RIN4) is a plasma membrane localized, evolutionarily conserved protein of 211 amino acids. Its sequence provides no clues to its function. RIN4 is required for RPM1-mediated disease resistance because it is required for RPM1 accumulation before infection. RIN4 is phosphorylated upon infection with P. syringae expressing either AvrB or AvrRpm1, though neither of these type III effectors has homology to known kinases (Lee et al., 2004). AvrB and AvrRpm1-dependent phosphorylation of RIN4 occurs in both RPM1 and rpm1 plants. These results suggested that RIN4 phosphorylation may result from the virulence activity of AvrB and AvrRpm1 and that this event leads to RPM1 activation when it is present (Mackey et al., 2002).

RIN4 is also involved in the activation of Resistance to P. syringae 2 (RPS2) (another CC-NB-LRR protein), with which it associates in vivo (Axtell and Staskawicz, 2003; Mackey et al., 2003). RPS2 confers resistance against P. syringae expressing the type III effector AvrRpt2 (Bent et al., 1994; Mindrinos et al., 1994). AvrRpt2 is a putative Cys protease (Axtell et al., 2003) that causes posttranscriptional disappearance of RIN4 (Axtell and Staskawicz, 2003; Mackey, 2003). Overexpression of RIN4 delays its disappearance in the presence of AvrRpt2 and, consequently, inhibits RPS2 activation. Thus, RIN4 disappearance is required for full RPS2 activation. A rin4 null mutation is lethal, and this lethality is rescued in a rin4 rps2 double mutant, indicating that RIN4 negatively regulates inappropriate activation of RPS2 (Mackey et al., 2003). We term this “inappropriate activation” to distinguish it from normal, AvrRpt2-dependent RPS2 activation (Belkhadir et al., 2004). Collectively, these data indicate that RIN4 is a target of multiple, unrelated bacterial type III effector proteins and that RIN4 associates with two different NB-LRR proteins. Both findings are consistent with the guard hypothesis for NB-LRR activation (Dangl and Jones, 2001).

Plant genes required for disease resistance were defined via genetic screens for loss of specific R functions (Glazebrook et al., 1997; Hammond-Kosack and Parker, 2003). Relevant to this work are nonspecific disease resistance 1 (NDR1) and RAR1, genes required for the function of various NB-LRR proteins. RAR1 is the founding member of the CHORD protein family, containing two novel zinc-coordinating domains (Shirasu et al., 1999; Muskett et al., 2002; Tornero et al., 2002). RAR1 may modulate NB-LRR protein levels (Tornero et al., 2002) through its association with HSP90 and other components of a signal-competent NB-LRR protein complex (Hubert et al., 2003; Liu et al., 2003; Lu et al., 2003) (reviewed in Holt et al., 2003; Shirasu and Schulze-Lefert, 2003; Belkhadir et al., 2004; Schulze-Lefert, 2004). RAR1 can associate with SGT1, a possible proteasome regulator required for the action of some, but not all, NB-LRR proteins (Austin et al., 2002; Azavedo et al., 2002; Tör et al., 2002). NDR1 modulates the intensity of signaling through specific NB-LRR proteins (Tornero et al., 2002). NDR1 may be a glycosylphatidylinositol (GPI) membrane anchored protein (Century et al., 1995, 1997; Coppinger et al., 2004). At least three CC-NB-LRR proteins, RPM1 (Boyes et al., 1998), RPS2 (Axtell and Staskawicz, 2003), and RPS5 (B. Holt, unpublished data), and their corresponding Avr proteins have been localized to the plasma membrane or to a membrane fraction (Nimchuk et al., 2000; Axtell and Staskawicz, 2003). Thus, NDR1 localization at the same subcellular address via a GPI anchor would place it in an excellent position to participate in the integration and transduction of NB-LRR signaling during infection.

Here, we assess whether RIN4 has any negative regulatory effect on inappropriate activation of RPM1, in addition to its requirement for RPM1 accumulation and its established negative regulatory effect on RPS2. We address the requirements for RAR1 and NDR1 for the inappropriate activation of RPS2 observed in the absence of RIN4. Finally, we address whether the virulence activities of AvrRpm1 and AvrRpt2 in susceptible plants lacking RIN4 are altered. Our results establish novel functions for RIN4 in the regulation of RPM1 and RPS2 activity and prompt a modification of the tenets of the guard hypothesis for disease resistance protein activation.

RESULTS

RPM1 Function Is Abrogated in rin4 Null Plants

We previously reported that a homozygous T-DNA insertion into the Rin4 open reading frame was embryo lethal. We demonstrated that the lethality of this rin4 null allele (hereafter, rin4; see Methods for allele designations of all mutants and transgenic lines used in this study) is largely suppressed in rin4 rps2 plants. This indicated that elimination of RIN4 results in inappropriate RPS2 activation (Mackey et al., 2003). We tested whether RPM1 is required for inappropriate RPS2 activation and the consequent lethal phenotype in selfed progeny from Rin4/rin4 RPS2/RPS2 rpm1/rpm1 plants. One-quarter of these plants died as embryos or early seedlings. Thus, the lethality in rin4 plants does not require RPM1 (data not shown).

We tested whether or not rpm1, like rps2, could suppress part or all of the rin4 lethal phenotype. Plants with reduced levels of Rin4 (rin4K-D; Rin4 knock-down plants because of an insertion in the Rin4 promoter: Wassilewskija-0 [Ws-0] background) (Mackey et al., 2002) are partially compromised for RPM1-mediated inhibition of bacterial growth because they accumulate
lowered levels of RPM1. We extended these analyses to RPM1 function in rin4 rps2 plants (Figure 1). P. syringae pv tomato (Pto) DC3000 (vector) grew to high levels by 3 d after infection on wild-type Columbia (Col-0) plants. Importantly, this growth was reduced reproducibly by 10-fold in rin4 rps2, indicating that these plants expressed enhanced basal disease resistance against Pto DC3000 (see below). Growth of Pto DC3000 expressing AvrRpm1, AvrB, or AvrRpt2 was inhibited on wild-type Col-0 plants as a result of RPM1 or RPS2 action, respectively. The growth of each strain was enhanced in rpm1 rps2 (Figure 1), as expected in the absence of the respective R proteins.

Importantly, the growth of Pto DC3000 (avrRpm1) (Figure 1) or Pto DC3000 (avrB) (data not shown) was the same in rin4 rps2 plants as in rpm1 rps2 plants, indicating a full loss of RPM1 function in the former plants, even though they are genotypically RPM1. Finally, the enhanced resistance against Pto DC3000 that we noted above in rin4 rps2 plants was not apparent against Pto DC3000 expressing avrRpm1 or avrRpt2 (Figure 1). Thus, these type III effectors (and avrB; data not shown) allow Pto DC3000 to overcome the enhanced basal disease resistance we observed in rin4 rps2 plants, presumably by suppressing an ectopic defense response (Figure 1).

**Enhanced Resistance against Pto DC3000 in rin4 Is Because of Ectopic Activation of Residual RPM1**

Numerous mutants exhibiting enhanced heightened resistance to pathogens also constitutively express pathogenesis-related (PR) genes as a result of activation of basal defense responses (Glazebrook et al., 1997; Lorrain et al., 2003). The enhanced resistance we observed in rin4 rps2 plants against Pto DC3000 (vector) indicated a possible constitutive expression of PR (cpr) phenotype (Bowling et al., 1994). Therefore, we analyzed PR1 protein expression as a convenient marker typical of cpr phenotypes (Figure 2A). We observed some residual constitutive PR1 protein accumulation in rin4 rps2 plants (Figure 2A). No PR1 expression was observed in Col-0, rpm1 rps2, or most importantly, rin4 rps2 rpm1 plants (Figure 2A). For comparison, and as demonstrated previously (Mackey et al., 2002), rin4K-D plants express constitutively high levels of PR1. Note, however, that the rin4K-D plants are in Ws-0, precluding direct comparison of PR1 levels in Col-0 and Ws-0. Nevertheless, our results in the isogenic Col-0 lines in Figure 2A demonstrate a low level of residual RPM1-dependent PR1 expression in rin4 rps2 plants. Ectopic RPM1 activation thus explains both the enhanced resistance to Pto DC3000 in rin4 rps2 and the loss of that enhanced resistance in rin4 rps2 rpm1 plants (Figure 1).

We also tested whether or not ectopic RPM1 activation could be enhanced by increasing the RPM1 dose in the context of lowered RIN4 levels represented in the rin4K-D plants. We doubled the RPM1 dose by crossing an isogenic RPM1-myc transgene (driven by the native RPM1 promoter) into rin4K-D plants. We probed protein blots with anti-RIN4, anti-myc, and anti-PR1 antibodies (Figure 2B). As previously noted, rin4K-D plants accumulated reduced levels of RIN4 compared with wild-type isogenic RPM1-myc plants (Figure 2B). Figure 2B also demonstrates, however, that rin4K-D (RPM1-myc) plants expressed significantly more PR1 than rin4K-D plants. The rin4K-D (RPM1-myc) plants also exhibited accentuated phenotypes relative to rin4K-D (data not shown). These included smaller stature, lower fertility, loss of apical dominance, and sporadic lesions (Mackey et al., 2002). By contrast, doubling the RPM1 dose in the RIN4 (RPM1-myc) control plants did not result in detectable PR1 expression (Figure 2B) or in any other macroscopic phenotype observed in rin4K-D. Thus, the additional copy of RPM1 enhances all aspects of the rin4K-D phenotype.

The level of PR1 expression in both rin4 rps2 and rin4K-D (RPM1-myc) plants was influenced by environment. Growth in 16-h days resulted in more PR1 expression compared with 8-h day conditions. This is consistent with our previous observation that rin4K-D plants show an exacerbated morphology when grown in long day conditions compared with short day conditions (Mackey et al., 2002). We also consistently observed a lower mobility of RIN4 in Ws-0 compared with Col-0 (Figure 2A). This lower mobility is a result of constitutive phosphorylation of RIN4 because phosphatase treatment resulted in increased mobility (data not shown).

**Figure 1. RPM1 Function Is Abrogated in rin4 Null Plants.**

Growth of the Pto DC3000 strains expressing the indicated type III effector genes, displayed on the right, was measured on wild-type and mutant Arabidopsis lines indicated at the bottom. Four-week-old plants were infiltrated with 10⁵ colony-forming units (cfu)/mL of the bacteria per area of leaf plotted on a log₁₀ scale for day 0 (open bars) and day 3 (closed bars) (see Methods). Error bars represent the standard deviation among four samples. This experiment is representative of four independent replicates. The absence of error bars indicates low errors. A one-way analysis of variance (ANOVA) test was applied to each pair of values, and P < 0.01 for rin4 rps2 inoculated with Pto DC3000 (vector) compared with all of the others (asterisk).
Figure 2. Residual RPM1 Is Sufficient for Constitutive Defense Response in rin4 Null Plants.

(A) Total protein extracts were prepared from wild-type Col-0, rpm1 rps2, rin4 rps2, rin4 rps2 rpm1, Ws-0, and rin4 knock-down (rin4K-D) plants. These extracts were subjected to anti-RIN4 (top, WB:RIN4) or anti-PR1 (middle, WB:PR1) protein gel blot analysis. Ponceau staining of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; bottom) was for confirmation of equal loading in each lane. This experiment is representative of at least
Collectively, the results in Figure 2 indicate that (1) when levels of RIN4 are reduced, residual RPM1 is activated inappropriately, and PR1 expression and enhanced resistance are consequently induced. (2) Wild-type RIN4 levels are necessary and sufficient for both the proper accumulation of RPM1 and for prevention of its inappropriate activation; hence, RIN4 negatively regulates RPM1. (3) The constitutive expression of PR1 in rin4K-D plants is because of the sum of inappropriate activation of both RPS2 and RPM1.

**RAR1 and NDR1 Are Differentially Required for Ectopic RPS2 Activation in rin4**

rps2 suppresses lethality in rin4 (Mackey et al., 2003). We addressed whether mutation in signaling components required for AvrRpt2-dependent activation of RPS2 could suppress the ectopic RPS2 activation in rin4. RAR1 and NDR1 are both required for RPS2 signaling and presumably act in the same pathway (see Introduction). We therefore followed lethality in selfed progeny from Rin4/rin4 rar1/rar1, and Rin4/rin4 ndr1/ndr1 plants (Figure 3A).

The rar1 mutation delayed rin4 lethality, and we were able to isolate rin4 rar1 plants. These plants had limited viability, were dwarfed relative to their Rin4 rar1 siblings by ~2 weeks of age, formed numerous dead cell lesions spontaneously, and died before 3 weeks of age (Figure 3B). We previously demonstrated that RPM1 accumulation is severely reduced in rar1 plants (Tornero et al., 2002). To address whether RPS2 levels were similarly affected, we crossed rar1 to a transgenic line carrying an HA-epitope tagged version of RPS2 (driven by the native promoter in rps2; Axtell and Staskawicz, 2003). This line expresses an accelerated HR and enhanced inhibition of bacterial growth compared with wild-type Col-0 after inoculation with Pto DC3000 (avrRpt2), presumably as a result of slight RPS2 protein overexpression (Axtell and Staskawicz, 2003). We PCR-selected a rar1 rps2 (RPS2-HA) triple homozygous line (see Methods). As with RPM1-myc, we detected severely reduced levels of RPS2-HA protein in rar1 rps2 (RPS2-HA) plants (Figure 3C). These results indicate that (1) RAR1 is required for accumulation of at least two CC-NB-LRR proteins, and (2) rar1 does not fully suppress the rin4 lethality because the residual RPS2 in rin4 rar1 plants remains ectopically activated. These results are consistent with a quantitative role for RAR1 in NB-LRR accumulation.

We did not recover any rin4 ndr1 plants in the analyzed progenies (Figure 3A). Thus, ndr1 cannot suppress inappropriate RPS2 activation in rin4, although it is clearly required for AvrRpt2-dependent RPS2 activation (Century et al., 1995). Additionally, there is no diminution of RPS2-HA levels in ndr1 rps2 (RPS2-HA) plants (Figure 3C).

RPS2-HA is a plasma membrane protein, and this localization is retained in the absence of RIN4 after infection with Pto DC3000 (avrRpt2) (Axtell and Staskawicz, 2003). NDR1 is a predicted GPI anchored protein (Coppinger et al., 2004). We tested whether NDR1 is responsible for RPS2 localization because RPS2 mislocalization could account for the differential NDR1 requirement during AvrRpt2-dependent RPS2 activation compared with its inappropriate activation in rin4. We fractionated crude lysates from rps2 (RPS2-HA), ndr1 rps2 (RPS2-HA), and rar1 rps2 (RPS2-HA) transgenic plants into total, soluble, and microsomal fractions and analyzed protein blots (Figure 4A). RPS2-HA remained localized in the microsomal fraction in ndr1 and rar1 plants. Thus, gross mislocalization of RPS2 cannot explain either the loss of AvrRpt2-dependent RPS2 activation in ndr1 or the differential requirement for NDR1 in the two modes of RPS2 activation. Collectively, the results in Figures 3 and 4 indicate that (1) NDR1 is either upstream or independent of the inappropriate RPS2 activation in rin4, and (2) NDR1 does not regulate RPS2 function by controlling its accumulation, as does RAR1, or its localization.

We conducted communoprecipitation experiments to test whether RIN4 also interacts with RPS2 in rar1 and ndr1 mutants (Figure 4B). We used rps2 (RPS2-HA), ndr1 rps2 (RPS2-HA), and rar1 rps2 (RPS2-HA) transgenic plants. Proteins immunoprecipitated with anti-RIN4 antisera were analyzed for RPS2-HA in protein blots. Neither ndr1 nor rar1 affected the ability of RIN4 to communoprecipitate RPS2-HA, despite the overall lower levels of RPS2-HA accumulating in rar1 (Figure 4B). The data presented in Figures 3 and 4 indicate that neither RAR1 nor NDR1 affects the mechanism of inappropriate RPS2 activation in rin4 plants, though RAR1 apparently dampens it by modulating RPS2 accumulation.

**Wild-Type Levels of NDR1 Are Sufficient to Transduce Enhanced RPS2 Function**

Our data indicate that NDR1 acts upstream or independent of inappropriate RPS2 activation in rin4. There is however a possible
alternative explanation for the inability of ndr1 to suppress rin4 lethality, where NDR1 would act downstream of RPS2 activation. NDR1 acts quantitatively during NB-LRR activation (see Introduction). There is obviously sufficient NDR1 in a wild-type plant to transduce a normal, AvrRpt2-driven RPS2 response. It might be that the quantity of signal flux during inappropriate RPS2 activation in rin4 is greater, or more sustained, than during infection. Thus, the signal flux during inappropriate RPS2 activation may overcome the normal requirement for NDR1 such that the lethal rin4 phenotype is generated via bypass in an ndr1 mutant.

To address this possibility, we took advantage of the accentuated RPS2 function in our rps2 (RPS2-HA) transgenic line (introduced above; Axtell and Staskawicz, 2003). This line should produce more flux through RPS2 during an AvrRpt2-driven response than the wild type. We established this point by comparing RPS2 function in rar1 rps2 (RPS2-HA) and ndr1 rps2 (RPS2-HA) to rar1 and ndr1 (Figure 5). Pto DC3000 (avrRpt2) growth was restricted in wild-type Col-0 and even more restricted in rps2 (RPS2-HA), reflecting enhanced RPS2 action as previously noted (Axtell and Staskawicz, 2003). Pto DC3000 (avrRpt2) grew to high levels on rps2. This growth was 90% reduced in rar1, indicating that the residual RPS2 in rar1 plants still functions. Importantly, Pto DC3000 (avrRpt2) growth was reduced by >99.5% in rar1 rps2 (RPS2-HA), indicating that the enhanced AvrRpt2-dependent RPS2 activation in this line is sufficient to partially overcome the lack of RAR1 in rar1 rps2.
The latter result argues against a bypass of even the enhanced signaling observed in wild-type levels of NDR1 are necessary and sufficient to mediate RPS2 stability or accumulation. Furthermore, they indicate that results are also consistent with a role for RAR1 in modulating the enhanced RPS2 signal was still fully NDR1 dependent. These

We used a weak pathogen strain, elimination of RIN4 would result in loss of that virulence activity. If RIN4 is the only target for AvrRpt2 when this type III effector but RIN4 Is Not the Only Target of AvrRpt2

acts as a virulence factor in its ability to enhance bacterial virulence in rpm1 plants (Mackey et al., 2002). Thus, RIN4 might be the target, or be a partner in a complex with the target(s) of the AvrRpm1 and AvrB virulence function(s). To study the relationship between the virulence activities of these type III effectors and RIN4, we tested whether the absence or overexpression of RIN4 alters the phenotypes associated with AvrRpm1 and AvrB in Arabidopsis

levels (Figure 7A). This growth was unaffected by the expression of RIN4 does not affect this virulence activity of AvrRpm1. We performed a similar set of experiments with Pma M6CΔE (avrRpm1) to grow reproducibly 10-fold more than Pma M6CΔE (vector) in rpm1 plants. This was observed on each rpm1 genotype tested, including rin4 rpm1 eps2 (Figure 7A). We conclude that the lack, or overexpression, of RIN4 does not affect this virulence activity of AvrRpm1.

We performed a similar set of experiments with Pma M6CΔE (avrB) (data not shown). Unlike AvrRpm1, AvrB is not able to promote pathogen growth on rpm1, though it can add to P. syringae virulence on susceptible soybean (Glycine max) genotypes (Ashfield et al., 1995). Altered levels of RIN4 did not alter the growth of this strain compared with Pma M6CΔE (vector) on any tested plant line (data not shown).

AvrB can cause a chlorotic response when expressed in rpm1, potentially indicative of its virulence activity (Nimchuk et al., 2000). We addressed whether modifications of RIN4 levels alter this phenotype. Figure 7B demonstrates that AvrB-dependent chlorosis in rpm1 is RIN4 independent. Furthermore, AvrB

(RPS2-HA). By contrast, the growth of Pto DC3000 (avrRpt2) was identical on nrd1 and nrd1 eps2 (RPS2-HA), demonstrating that the enhanced RPS2 signal was still fully NDR1 dependent. These results are also consistent with a role for RAR1 in modulating RPS2 stability or accumulation. Furthermore, they indicate that wild-type levels of NDR1 are necessary and sufficient to modulate even the enhanced signaling observed in rar1 eps2 (RPS2-HA). The latter result argues against a bypass of NDR1 function during inappropriate RPS2 activation in rin4.

RIN4 Levels Modulate AvrRpt2 Virulence Function

but RIN4 Is Not the Only Target of AvrRpt2

If RIN4 is the only target for AvrRpt2 when this type III effector acts as a virulence factor in eps2, then it could be the case that elimination of RIN4 would result in loss of that virulence activity. We used a weak pathogen strain, P. syringae pv maculicola (Pma) M6CΔE (Rohmer et al., 2003; see Methods), to examine the contribution of AvrRpt2 to bacterial virulence on plants with altered levels of RIN4. Note that we observed only a weak RPS2-dependent inhibition of bacterial growth with Pma M6CΔE (avrRpt2) at low bacterial doses (Figure 6A). However, using a higher titer of bacteria, we observed consistently RPS2-mediated HR (data not shown). The weak RPS2-mediated inhibition of bacterial growth is likely because of the weak intrinsic virulence of Pma M6CΔE.

We reproducibly observed a very slight increase in the virulence of Pma M6CΔE (avrRpt2) on eps2 compared with Col-0 (Figure 6). AvrRpt2 delivered from Pma M6CΔE promotes increased bacterial growth in rin4 eps2 plants compared with eps2 plants (Figure 6A). This enhanced virulence function of AvrRpt2 is reversed in eps2 plants that overexpress RIN4 (OxRIN4 eps2 plants; Mackey et al., 2003) (Figure 6A). These data indicate that (1) RIN4, in a formal sense, negatively regulates one or more AvrRpt2 virulence activities; (2) wild-type levels of RIN4 are apparently saturating for this negative regulation; (3) RIN4 is not required for this AvrRpt2 virulence activity.

The Absence of RAR1 and NDR1 Enhances AvrRpt2 Virulence Function(s)

AvrRpt2 is able to promote the virulence of Pto DC3000 by suppressing plant defenses downstream or independently of salicylic acid (SA)-dependent basal defenses (Chen et al., 2004). RAR1 and NDR1 can regulate basal plant defense (see Introduction). We therefore addressed the contribution of RAR1 and NDR1 to AvrRpt2 virulence activities by inoculating Pma M6CΔE (avrRpt2) onto rar1 eps2 and nrd1 eps2 (Figure 6B). Again, Pma M6CΔE (avrRpt2) grew reproducibly to higher titers on eps2 than did Pma M6CΔE (vector), indicative of an AvrRpt2 virulence function. This was enhanced in rin4 eps2, as in Figure 6A. Importantly, AvrRpt2 promoted more bacterial growth in rar1 eps2 and nrd1 eps2 compared with eps2 (Figure 6B). These results indicate that RAR1 and NDR1 negatively regulate one or more AvrRpt2 virulence activities, presumably via their functions in the induction of basal defense.

RIN4 Is Not the Only Target of AvrRpm1 and AvrB in Arabidopsis

The ability of AvrRpm1 and AvrB to interact with RIN4 and to induce its phosphorylation may contribute to their ability to enhance bacterial virulence in rpm1 plants (Mackey et al., 2002), which are resistant to RPM1 and RPS2 independent (data not shown). Pma M6CΔE (avrRpm1) growth in wild-type Col-0 was significantly reduced, because of RPM1 action, compared with growth in rpm1 eps2, rin4 rpm1 eps2, or OxRIN4 rpm1 (Mackey et al., 2003). Pma M6CΔE (vector) grew to intermediate levels (Figure 7A). This growth was unaffected by the expression of Pma M6CΔE (avrRpm1) in rpm1 plants, which is RIN4 independent. Furthermore, AvrB

Growth of Pto DC3000 (avrRpt2) was measured on wild-type and mutant Arabidopsis lines indicated at the bottom. Bacterial growth was measured as described in the legend of Figure 1. Error bars represent the standard deviation among four samples, and this experiment is representative of two independent replicates. The absence of error bars indicates low errors.
accumulates in a RIN4-independent manner (the modest difference in the levels of AvrB in this experiment is sporadic and does not correlate with expression of RIN4; data not shown). The results presented in Figure 7 indicate that whereas RIN4 is certainly an avirulence target for both AvrRpm1 and AvrB, it is not their only virulence target. Alternatively, a direct requirement of RIN4 for the virulence activities of AvrRpm1 and AvrB cannot be measured in our assays.

**DISCUSSION**

This work was aimed at clarifying the role of the Arabidopsis RIN4 protein in the control of RPM1 and RPS2 activation. We further tested whether RIN4 is the unique target of AvrRpm1, AvrB, and AvrRpt2 when these type III effectors function as virulence factors. We show that RIN4 has a negative regulatory function that blocks the inappropriate activation of RPM1 in addition to a similar regulatory function previously established for RIN4 in RPS2 activation (Axtell and Staskawicz, 2003; Mackey et al., 2003). We propose that wild-type levels of RIN4 are required to maintain RPM1 and RPS2 in a non-signaling configuration. We demonstrate that inappropriate RPS2 activation, leading to lethality in *rin4* plants, is quantitatively dependent on RAR1 but independent of NDR1. The latter observation differentiates this mode of RPS2 activation from its normal, AvrRpt2-driven activation and strongly indicates that RIN4 functions at, downstream, or independent of NDR1 to control RPS2 activity. We also demonstrate that RIN4 is not the only target of AvrRpm1, AvrB, and AvrRpt2 with respect to the virulence activities of these three type III effectors. Surprisingly, RIN4 negatively regulates at least one virulence activity of AvrRpt2. We propose that *P. syringae* type III effector proteins may frequently have multiple targets in susceptible plants. Their manipulation of a subset of these targets (one, in fact) is demonstrably sufficient for...
activation of at least RPM1 and RPS2. Our data extend the notion that NB-LRR proteins monitor the activities of type III effector proteins expressed by pathogenic bacteria and have implications for the evolution of the plant immune system.

RIN4 Negatively Regulates Inappropriate RPM1 Activation

The rin4 lethality was largely suppressed in a rin4 rps2 double mutant, proving that inappropriate RPS2 activation is negatively regulated by RIN4 (Mackey et al., 2003). Yet residual signaling in rin4 rps2 is sufficient to drive enhanced basal defense against Pto DC3000 (Figure 1) and PR1 expression (Figure 2A). The residual RPM1 present in rin4 rps2 is responsible for these phenotypes because they are eliminated in rin4 rps2 rpm1 triple mutants. Note that this residual RPM1 is not competent to transduce AvrRpm1- or AvrB-dependent signals (Figure 1; data not shown). Thus, RIN4 also negatively regulates inappropriate RPM1 activity. Wild-type RIN4 levels are apparently saturating for maintaining RPM1 in an inactive state because neither a doubling of the RPM1 dose (Figure 2B) nor RIN4 overexpression (Mackey et al., 2002) affects RPM1 function. RPM1 was inappropriately active in wild-type plants when overexpressed (Leister and Katagiri, 2000), possibly because of an elevated RPM1/RIN4 ratio.

Four related models can explain these data. (1) RPM1 is activated in rin4 plants because RIN4 is a negative regulator of RPM1 activation, and that regulation is lacking. The lowered RPM1 levels we observed in rin4K-D (Figure 2B) would then be a consequence of RPM1 disappearance following its activation

Figure 7. RIN4 Is Not the Only Virulence Target for AvrRpm1 and AvrB in Arabidopsis.

(A) Growth of Pma M6CΔE carrying empty vector or avrRpm1 indicated at bottom was measured on the genotypes indicated at top. Four-week-old plants were infiltrated with 10^4 cfu/mL and the number of bacteria per area of leaf plotted on a log_10 scale for day 0 (open bars) and day 3 (closed bars) (see Methods). Error bars represent the standard deviation among four samples, and this experiment is representative of three independent replicates. The absence of error bars indicates insignificant differences.

(B) Agrobacterium tumefaciens carrying empty vector or dexamethasone (DEX) inducible avrB-HA as indicated at bottom were inoculated onto leaves of various genotypes indicated at top, at 10^10 cfu/mL. Leaves were sprayed 24 h postinoculation with DEX (20 μM) and photographed 96 h after that. Total protein extracts were prepared 96 h after DEX and subjected to anti-HA protein gel blot analysis.
(Boyes et al., 1998). (2) Specific RPM1 activation might require the physical interaction of AvrRpm1 or AvrB with RIN4 (Mackey et al., 2002) or a RIN4-containing complex, and that interaction could be disrupted when residual RPM1 misaccumulates in the absence of RIN4. (3) Residual, activated RPM1 might lose its responsiveness to AvrRpm1 and AvrB. This would be analogous to CARD15/NOD-2 variants that ectopically activate the NF-κB pathway but lose responsiveness to lipopolysaccharide and subsequent, appropriate NF-κB activation (Tanabe et al., 2004).

(4) RPM1 simply might not accumulate enough in the absence of RIN4 to allow a robust AvrRpm1- or AvrB-specific response in rin4 plants. This possibility, though, is inconsistent with the established notion that NB-LRR protein activation requires a lower threshold of signal than does activation of basal defense (Tao et al., 2003).

Lowering of RPM1 levels, however, is not necessarily accompanied by activation of basal defense. Arabidopsis rar1 mutants accumulate very low levels of RPM1 but display normal susceptibility to Pto DC3000 (Tornero et al., 2002), rather than the enhanced resistance that we observed in rin4 rps2. Arabidopsis aths90.2 mutants also express similar RPM1 reduction that is correlated with a diminution of RPM1 function (Hubert et al., 2003). Thus, RPM1 is destabilized in atr1 or aths90.2 without concomitant activation of basal defense. This is consistent with a proposed function of RAR1/SGT1/HSP90 for assembly of signal-competent RPM1 upstream of any activation (Hubert et al., 2003; Belkhadir et al., 2004).

Activation of the Resistance to Potato Virus X NB-LRR protein is dependent on finely tuned intramolecular interactions (Moffett et al., 2002; Rathjen and Moffett, 2003; Belkhadir et al., 2004). Intramolecular interactions are often conditioned and modulated by intermolecular interactions (Djordjevic et al., 1998; Autiero et al., 2003). The inappropriate RPM1 activation in rin4 rps2 might also be because of the consequences of intramolecular changes induced by the absence of normal interactions between RPM1, RIN4, and other putative components. This model is consistent with a possible requirement for RIN4 phosphorylation during AvrRpm1- or AvrB-induced activation of RPM1 because phosphorylation events are known to induce changes in protein–protein interactions (Djordjevic et al., 1998).

**Inappropriate RPS2 Activation Is Independent of NDR1 and Modulated by RAR1**

NDR1 is required for AvrRpt2-driven activation of RPS2. It was previously shown that NDR1 is not required for the AvrRpt2-induced disappearance of RIN4 (Axtell and Staskawicz, 2003). Here, we show that NDR1 is not required for RPS2 accumulation, gross localization, or association with RIN4. Thus, three important requirements for the RIN4-dependent activation of RPS2 by AvrRpt2 are NDR1 independent. These results corroborate our genetic demonstration that ndr1 is not able to suppress inappropriate RPS2 activation in rin4. Thus, the events leading to either AvrRpt2-driven RPS2 activation or its inappropriate activation in rin4 are separable. Very little is known about how NDR1 functions in NB-LRR activation. Based on our data, we propose (1) that NDR1 does not affect NB-LRR stability or NB-LRR localization and (2) that NDR1 is not required for signaling downstream of NB-LRR protein activation. Instead, we envision that NDR1 functions upstream of NB-LRR activation by various pathogens.

RAR1 is required for RPS2 and RPM1 signaling in Arabidopsis (see Introduction). The accumulated data indicates that RAR1 limits defense signal flux, perhaps by modulating NB-LRR stability or accumulation (Tornero et al., 2002). Our results indicate that RAR1 also modulates RPS2-HA accumulation (Figure 3). Heightened RPS2 signaling capacity, presumably achieved by slight overexpression, can partially overcome the lack of RAR1 in rar1 rps2 (RPS2-HA) plants (Figure 6). We propose that RAR1 acts generally on NB-LRR proteins by controlling their accumulation and/or stability and not by modulating a common downstream signal.

**AvrRpt2, AvrRpm1, and AvrB Manipulate Basal Defense**

The enhanced resistance against Pto DC3000 in rin4 rps2 plants is abrogated when the bacteria express AvrRpm1 (Figure 1), AvrRpt2 (Figure 1), or AvrB (data not shown). Thus, these proteins can presumably suppress the basal defense activated in rin4 rps2. Our findings are also consistent with recent data indicating that AvrRpt2 acts as a virulence factor downstream or independent of SA accumulation (Chen et al., 2004) and with recent data suggesting that a variety of *P. syringae* type III effectors manipulate plant basal defense responses (Abramovitch and Martin, 2004).

*Pto* DC3000 (avrRpm1) and *Pto* DC3000 (avrRpt2) suppress the enhanced basal resistance against *Pto* DC3000 observed in rin4 rps2 (Figure 6). These data clearly indicate that RIN4 is either not a virulence target or not the only target for AvrRpm1 and AvrRpt2 in rin4 rps2. In fact, AvrRpt2-dependent virulence is enhanced in rin4 rps2 (Figure 6; see below). The enhancement of AvrRpt2-dependent virulence on rin4 rps2 was also observed when it was delivered from *Pma* M6CΔE (Figure 6). Because we did not observe enhanced resistance against *Pma* M6CΔE on rin4 rps2, AvrRpt2 may enhance the growth of this strain in a manner distinct from its function in *Pto* DC3000.

**rar1 and ndr1 Mutations Enhance AvrRpt2 Virulence Function(s)**

*ndr1* plants are impaired in basal defense responses (our unpublished data). AvrRpt2 was recently shown to promote virulence in rps2 by suppressing defense gene expression downstream or independent of SA (Chen et al., 2004). We extend these results by demonstrating that ndr1 rps2 and rar1 rps2 support significantly more AvrRpt2-dependent *Pma* M6CΔE growth than rps2 (Figure 6). Hence, the loss of basal defense signaling normally induced via NDR1 and RAR1 enhances the observed effect of AvrRpt2. We therefore propose that there are multiple basal defense pathways that are downstream or independent of SA. Some of these are targeted by AvrRpt2, whereas others are NDR1 and/or RAR1 dependent.

**RIN4 Is Not the Only Target of AvrRpm1, AvrRpt2, or AvrB**

If each type III effector has a specific, single host target, then it follows that elimination of that target would diminish pathogen
virulence. We hypothesized that elimination of RIN4 in the rin4 rps2 rpm1 triple mutant would allow us to determine whether the known virulence function of AvrRpm1 requires RIN4. Our data clearly indicate that AvrRpm1 virulence function and AvrB-dependent chlorosis are maintained (Figure 7) and that AvrRpt2 function is unexpectedly enhanced (Figure 6) in rin4 rps2. Thus, although RIN4 is assuredly a target of AvrRpm1, AvrB, and AvrRpt2 (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003), it is not the only target for any of them. We propose that type III effectors from P. syringae, like those from Shigella flexneri, have multiple host cellular targets (Hilbi et al., 1998; Lafont et al., 2002).

We established that, surprisingly, RIN4 negatively regulates virulence mediated by AvrRpt2 (Figure 6). AvrRpt2 encodes a probable Cys protease, and it was proposed that this activity destabilizes RIN4 or a RIN4-containing complex (Axtell et al., 2003). Our observations of (1) increased bacterial growth mediated by AvrRpt2 on rin4 rps2 plants and (2) reversal of that effect by RIN4 overexpression fit a model where a limited number of translocated AvrRpt2 molecules could operate on several cellular substrates. We envision that the specific activity of the AvrRpt2 protease for other substrates is increased in rin4 plants. As a result, the other targets are neutralized more quickly or more efficiently, and the fitness of the bacteria on rin4 plants is increased. Alternatively, RIN4 regulates a basal defense pathway that is possibly targeted by AvrRpt2.

Is RIN4 the Only Bacterial Type III Effector Target Guarded by RPM1 and RPS2?

RIN4 is evolutionarily conserved based in at least rice (Oryza sativa), maize (Zea mays), tobacco (Nicotiana tabacum), tomato (Lycopersicon esculentum), and potato (Solanum tuberosum) (D. Desveaux, unpublished data). The functional association of two NB-LRR proteins (RPM1 and RPS2) with RIN4 in Arabidopsis, combined with RIN4’s conservation, raises the possibility that RIN4 regulates defense responses in those plant species as well. Our work indicates though that RIN4 is not the only virulence target of AvrRpm1, AvrB, and AvrRpt2. It is thus legitimate to question whether RPS2 and RPM1 monitor the homeostasis of RIN4 alone or, alternatively, of RIN4 and a subset of other AvrRpm1, AvrB, and AvrRpt2 targets.

Ashfield et al. (2004) recently demonstrated that the NB-LRR protein that recognizes AvrB (but not AvrRpm1) in soybean, Rpg1-b, is not the closest ortholog of RPM1. They further showed that AvrRpt2 could interfere with AvrB-dependent activation of Rpg1-b, consistent with results in Arabidopsis (Ritter and Dangl, 1996) but that this interference may not be because of the AvrRpt2-dependent elimination of RIN4, as observed in Arabidopsis (Mackey et al., 2003). Furthermore, they saw no clear AvrB-dependent mobility changes in anti-RIN4 cross-reacting bands in soybean protein extracts. Thus, although more work remains to be done, it may be that Rpg1-b is not associated with RIN4, but rather with another host target of both AvrB and AvrRpt2.

The evolution of a single NB-LRR protein guarding any of the several potential targets of a given virulence factor is demonstrably sufficient to initiate successful disease resistance against pathogen strains expressing that virulence factor. Particularly effective virulence factors would presumably spread through the pathogen population at frequencies balanced by the rate of evolution of NB-LRR proteins that detect their action. This might drive evolution of multiple NB-LRR genes whose products recognize the action of a single virulence factor at different targets.

There may be, however, fundamental evolutionary pressures limiting the number of targets that a particular NB-LRR protein can simultaneously guard. The first is structural. If the various virulence factor targets are divergent, a single NB-LRR protein might not be able to productively interact with all of them. The second may be reflected by the fact that maintenance of RPM1 expression in Arabidopsis results in a substantial fitness cost for the plant (Grant et al., 1998; Stahl et al., 1999; Bergelson et al., 2001). This might be generally true because constitutive NB-LRR activation results in cell death (Hu et al., 1996; Collins et al., 1999; Shirano et al., 2002; Zhang et al., 2003). Thus, a potential explanation for the apparently limited number of AvrRpm1, AvrB, and AvrRpt2 targets that are effectively guarded by RPM1 and RPS2 could be an inherent fitness cost associated with increasing NB-LRR expression levels. An increase in the number of host targets guarded by a particular NB-LRR protein might result in an increase in overall levels of that protein and an attendant fitness cost.

METHODS

Pseudomonas syringae

Pto DC3000 carrying either pVSP61 or derivatives of this plasmid containing avr genes have been described (Mackey et al., 2002, 2003), and Pma M6CΔE is a derivative of a weakly virulent isolate of P. syringae pv maculicola (Rohmer et al., 2003). Bacterial growth in plant leaves was measured by two methods. Figure 1 was done by inoculating 4-week-old plants with 10^5 cfu/mL. In Figures 5 and 6, 4-week-old plants were inoculated with 10^6 cfu/mL. For each sample, four leaf discs were pooled four times per data point (16 leaf discs total). Leaf discs were bored from the infiltrated area, ground in 10 mM MgCl2, and serially diluted to measure bacterial numbers.

Protein

Total protein extracts were prepared and cell fractionation and commounprecipitation assays performed as described by Mackey et al. (2002, 2003). Anti-RIN4 serum was used at a dilution of 1:5000. The anti-PR-1 serum (gift of Robert A. Dietrich, Syngenta, Research Triangle Park, NC) was used at a dilution of 1:10000. The anti-RD28 and anti-APX (gifts of Maarten Chrispeels and Daniel Kliebenstein, respectively) antibodies were used at a dilution of 1:5000. Detection of HA and myc epitope tags was with supernatants from cultures of hybridoma 9E10 monoclonal anti-HA antibody (Roche, Indianapolis, IN), at a dilution of 1:1000, and the hybridoma 9E10 monoclonal anti-myc antibody, at a dilution of 1:10 (Boyes et al., 1998).

Plants and Mutant Construction

The following plant genotypes were used in this work: rps2-101C is an allele of RPS2 in Col-0 with a stop codon after amino acid 235 (Bent et al., 1994); rpm1-3 is an allele of RPM1 with a stop codon after amino acid 87 (Grant et al., 1995). The rin4 null is a T-DNA insertion in the RIN4 open
reading frame in Col-0 (Mackey et al., 2003). The rin4K-D is a T-DNA insertion in the promoter of RIN4 in Ws-0 (Mackey et al., 2002). The triple mutant rin4 rpm1 rps2 was constructed like the rin4 rps2 double mutant described by Mackey et al. (2003) using the Col-0 rin4 null allele. The RPM1 PCR product was digested with EcoRV, which cut the wild type, but not rpm1-1, into a doublet. The rin4K-D RPM1-myc line was made by crossing a Ws-0-based RPM1-myc transgenic line to the Ws-0 rin4K-D plants. The rin4 K-D plants were used as a pollen source. RPM1-myc was followed by hygromycin resistance and rin4K-D was followed phenotypically. The RPM1-myc and rin4K-D RPM1-myc plants in the Ws-0 background have both an endogenous and the transgenic copy of RPM1. Mutant alleles of the ndr1-1 null (Century et al., 1997) and the premature stop in rar1-21 (Tomore et al., 2002) were PCR selected using primers, and conditions are available on request.

nrdr1 rps2 and nrd1 rps2 RPS2-HA plants were selected from a cross between nrd1 and rps2 RPS2-HA (Axtell et al., 2003). A degenerate cleaved amplified polymorphic sequence marker able to identify plants carrying the rps2 mutation was run on DNA of F2 individuals selected to be homozygous for the ndr1 mutation. Individuals carrying only the rps2 allele were confirmed in the next generation to be nrd1 rps2. Thirty-six progeny from individuals appearing as heterozygous for the rps2 mutation in the F2 generation were rechecked for homozygosity of both the native mutant version of rps2 and the transgenic wild-type version of RPS2 using the same marker. Those families selected to be nrd1 rps2 RPS2-HA were further confirmed by anti-HA protein gel blot analysis. rar1 rps2 RPS2-HA and rar1 rps2 plants were identified in the same fashion.

Agrobacterium tumefaciens Transient Expression Assays

Two-milliliter Agrobacterium cultures were grown overnight at 30°C in YEB (5 g of bacto beef extract, 1 g of bacto yeast extract, 5 g of bacto peptone, 5 g of sucrose, and 2 mM MgSO4, pH 7.2, per liter) containing 100 μg/mL each of rifampicin, kanamycin, and gentamycin for strain GV3101. The next day, 150 μL of saturated culture was inoculated into 3 mL of YEB plus antibiotics and grown for 13 h. Two milliliters were collected and resuspended in 3 mL of Agrobacterium induction medium (10.5 g of K2HPO4, 4.5 g of KH2PO4, 1 g of (NH4)2SO4, 0.5 g of NaCitrate), 1 mM MgSO4, 1 g of glucose, 1 g of fructose, 4 mL of glycerol, 10 mM Mes, pH 5.6, and 50 μg/mL of acetosyringone), grown at 2°C for 5 to 7 h, collected, and resuspended in infiltration medium (half-strength Mes-Mes) to an OD600 of 0.4. The underside of 3-week-old leaves was inoculated using a needleless syringe. Plants were grown in 120 μL of light and sprayed with 20 μM DEX (Sigma, St. Louis, MO). To inductively express AvrB in planta, the gene with a C-terminal HA-tag was cloned into pTA7002 (Aoyama and Chua, 1997; Nimchuk et al., 2000).

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Downstream or Independent of the NDR1 Signal Modulator and Is Not Required for the Virulence
Functions of Bacterial Type III Effectors AvrRpt2 or AvrRpm1
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