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Two *Pseudomonas syringae* Type III Effectors Inhibit RIN4-Regulated Basal Defense in *Arabidopsis*

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Summary

Plant cells have two defense systems that detect bacterial pathogens. One is a basal defense system that recognizes complex pathogen-associated molecular patterns (PAMPs). A second system uses disease-resistance (R) proteins to recognize type III effector proteins that are delivered into the plant cell by the pathogen's type III secretion system. Here we show that these two pathways are linked. We find that two *Pseudomonas syringae* type III effectors, AvrRpt2 and AvrRpm1, inhibit PAMP-induced signaling and thus compromise the host's basal defense system. RIN4 is an *Arabidopsis* protein targeted by AvrRpt2 and AvrRpm1 for degradation and phosphorylation, respectively. We find that RIN4 is itself a regulator of PAMP signaling. The R proteins, RPS2 and RPM1, sense type III effector-induced perturbations of RIN4. Thus, R proteins guard the plant against type III effectors that inhibit PAMP signaling and provide a mechanistic link between the two plant defense systems.

Introduction

Plants use an active immune system to combat pathogenic challengers (Jones and Takemoto, 2004). Complex surveillance systems detect various molecules associated with infection and initiate defensive responses (Nimchuk et al., 2003; Nurnberger et al., 2004). Distinct branches of the innate immune system respond to two general classes of pathogen-derived molecules, *p*athogen-*a*ssociated *m*olecular *p*atterns (PAMPs) and, in the case of pathogenic bacteria, type III effector proteins. Here, we show that one branch of the plant immune system responds to a pathogen by detecting the pathogen's perturbation of another branch.

PAMPs are recognized by the innate immune system of animals and plants. Because PAMPs are often required for pathogen viability and are frequently invariant among a broad class of pathogens, they make ideal elicitors for "nonself" surveillance systems. In animals, PAMP signaling is critical to combating a variety of pathogenic threats and for full engagement of the adaptive immune response (Qureshi and Medzhitov, 2003). In plants, a variety of PAMPs (e.g., chitin, ergosterol, and a transglutaminase from fungi, and lipopolysaccharide and flagellin from bacteria) stimulate plant-encoded PAMP receptors (Nurnberger et al., 2004). Genes encoding two PAMP receptors have been identified (Gómez-Gómez and Boller, 2000; Ron and Avni, 2004). The encoded proteins both have extracellular LRRs and one, the FLS2 protein, has a cytoplasmic kinase domain. Intriguingly, the leucine-rich repeats (LRRs) of FLS2 are closely related to those of Toll-like receptors involved in the perception of PAMPs by animals (Nurnberger et al., 2004). Upon recognition of a 22 amino acid peptide derived from the highly conserved amino terminus of flagellin (called flg22), FLS2 induces a suite of defense responses, including map kinase signaling (Asai et al., 2002), transcriptional activation (Navarro et al., 2004), and deposition of callose, a putative physical barrier at the site of infection (Gómez-Gómez et al., 1999). The plant responses induced by flg22 and other PAMPs are referred to as "basal" defenses.

Bacterial pathogens deploy type III effector proteins that promote their virulence. Not surprisingly, plants have evolved disease-resistance (R) proteins that mediate specific recognition of these proteins, leading to accelerated and amplified defense responses. Numerous R proteins have been identified; the majority contain a nucleotide binding site and LRRs and are apparently intracellular (Dangl and Jones, 2001; Martin et al., 2003). These resemble NOD proteins, a second class of animal proteins involved in the perception of PAMPs (Inohara and Nunez, 2003). R protein-mediated recognition of pathogen-derived type III effectors results in activation of defense responses that frequently culminate in host cell death, a dramatic outcome termed the hypersensitive response (HR; Heath, 2000). Though qualitatively similar to defense responses induced by PAMP receptors, those responses induced by R proteins are typically stronger and more rapid (Asai et al., 2000; Navarro et al., 2004; Tao et al., 2003). A given type III effector does not always induce these responses because the distribution of functional R proteins is highly polymorphic and thus plant strain (cultivar) specific (Lehmann, 2002). When the cognate R protein is lacking, an effector does not induce resistance and instead carries out its virulence function.

One virulence function of type III effectors is to help the pathogen avoid or overcome induced defense responses (Chang et al., 2004). *Pseudomonas syringae*

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are gram negative, plant pathogens that use a type three secretion system (TTSS) to deliver up to 40 type III effector proteins into host cells (Fouts et al., 2002; Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). Mutations that disrupt the TTSS and thereby prevent delivery of all type III effectors, severely compromise growth of the bacteria (Collmer et al., 2000; Deng et al., 1998). Collectively, the type III effectors make critical contributions to virulence of the bacteria. Numerous individual type III effector proteins do so by inhibiting host defense responses, including cell death associated with the HR (Espinosa and Alfano, 2004). Type III effectors can also inhibit basal defenses of the plant (Jakobek et al., 1993; Keshavarzi et al., 2004). For example, the bacterial type III effector AvrPto can block callose deposition induced by TTSS-deficient *P. syringae* (Hauck et al., 2003). Because callose deposition is also induced by FLS2 upon recognition of flg22 (Gómez-Gómez et al., 1999), it was proposed that AvrPto and possibly other type III effectors inhibit PAMP-induced defense responses.

Some R proteins recognize effectors indirectly. Specifically, perturbations induced by type III effector proteins, and not the type III effector proteins per se, are recognized by these R proteins. This idea is described by the "guard" hypothesis (Dangl and Jones, 2001; van der Biezen and Jones, 1998). Two tenets of this hypothesis are that (1) a given effector protein has a target(s) in the host independent of the corresponding R protein, and (2) by manipulating this target(s) the effector produces a perturbation that is, in turn, recognized by the corresponding R protein. RIN4 from *Arabidopsis* is a seminal example of a host target of type III effectors that is "guarded" by R proteins. Two unrelated type III effectors, AvrRpm1 and AvrB, interact with and induce phosphorylation of RIN4 (Mackey et al., 2002). The perturbation of RIN4 by AvrRpm1 or AvrB is hypothesized to induce the activity of their cognate R protein, RPM1. A third effector, AvrRpt2, a protease, also targets RIN4 and induces its posttranscriptional disappearance (Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003). Disappearance of RIN4 activates RPS2, the cognate R protein of AvrRpt2 (Axtell and Staskawicz, 2003; Mackey et al., 2003). These and other examples (Kruger et al., 2002; Shao et al., 2003) indicate that some R proteins recognize pathogens by perceiving effector-induced perturbations of the "guarded" proteins. A third, unproven tenet of the "guard" hypothesis is that the perturbations recognized by R proteins result from the virulence-promoting activity of type III effector proteins. Our results demonstrate that some type III effector proteins inhibit plant defenses by targeting proteins that regulate PAMP-induced defense signaling. These findings are consistent with the third tenet, and thus provide further support for the guard hypothesis.

Both AvrRpt2 and AvrRpm1 can contribute to bacterial virulence in plants lacking the respective R proteins, RPS2 and RPM1 (Chen et al., 2000; Ritter and Dangl, 1995). We studied the perturbations induced by AvrRpt2 and AvrRpm1 in these plants. Each effector inhibits defense signaling induced by FLS2 and other putative PAMP receptors. RIN4 is a negative regulator of PAMP signaling; PAMP-induced defense responses are inhibited in plants overexpressing RIN4, while these responses are enhanced in plants lacking RIN4. The effect of AvrRpt2, AvrRpm1, or RIN4 levels on plant defense correlates with the growth of *P. syringae* in assays that reveal the contribution of PAMP signaling to overall resistance. We show that callose deposition resulting from PAMP signaling is dependent on the *Arabidopsis* callose synthase gene, *PMR4*, and that PMR4 positively contributes to resistance against *P. syringae*. Our results indicate that (1) AvrRpt2 and AvrRpm1 inhibit PAMP-induced signaling by perturbing RIN4 and associated proteins; (2) RIN4 functions, independent of AvrRpt2, AvrRpm1, RPM1, and RPS2, to regulate PAMP-based defense signaling; and (3) RPM1 and RPS2 "guard" RIN4 to detect pathogens that disrupt PAMP-based immunity by perturbing RIN4 and associated proteins. This work provides further support for the guard hypothesis and establishes a fundamental relationship between PAMP- and effector-induced defense systems in the plant.

Results

AvrRpt2 and AvrRpm1 Enhance the Growth of TTSS-Deficient Bacteria

Mutation of a structural gene of the TTSS (*hrcC*) renders *P. syringae* pv. *tomato* (Pto) strain DC3000 incapable of injecting type III effector proteins into host cells. Pto DC3000*hrcC*, like its wild-type counterpart, displays PAMPs (such as flagellin and LPS). Basal defenses induced by these PAMPs likely contribute to the poor growth of this strain in planta (Collmer et al., 2000). It was previously shown that AvrPto, a type III effector encoded by Pto DC3000, expressed inside the cells of the plant could enhance the growth of Pto DC3000*hrcC* (Hauck et al., 2003). We determined whether other bacterial type III effectors could similarly enhance the growth of these TTSS-deficient bacteria (Figure 1A).

To test the contribution of individual type III effectors, Pto DC3000*hrcC* was inoculated into transgenic *Arabidopsis* plants in which expression of either AvrRpt2 or AvrRpm1 was induced with dexamethasone (Dex; see Experimental Procedures). These transgenic lines lack the R protein responsive to the expressed type III effector (plants expressing AvrRpt2 are *rps2* and the plants expressing AvrRpm1 are *rpm1*). Pto DC3000*hrcC* grew only 5-fold in 6 days on control plants but grew better on *rps2* plants expressing AvrRpt2, consistent with recently reported results (Chen et al., 2004). Importantly, Pto DC3000*hrcC* grew more than 1000-fold in 3 days on *rpm1* plants expressing AvrRpm1. The plant tissue was collapsed by day 6, and no measurement could be made. The level of growth of Pto DC3000*hrcC* in plants expressing AvrRpm1 approaches the level of growth of wild-type Pto DC3000 on wild-type plants in this assay (data not shown). Thus, AvrRpt2 (weakly) and AvrRpm1 (strongly) enhance the growth of TTSS-deficient *Pseudomonas*.

AvrRpt2 and AvrRpm1 Inhibit PAMP-Induced Defense Responses

We hypothesized that AvrRpt2 and AvrRpm1 enhance the growth of Pto DC3000*hrcC* by suppressing PAMP-

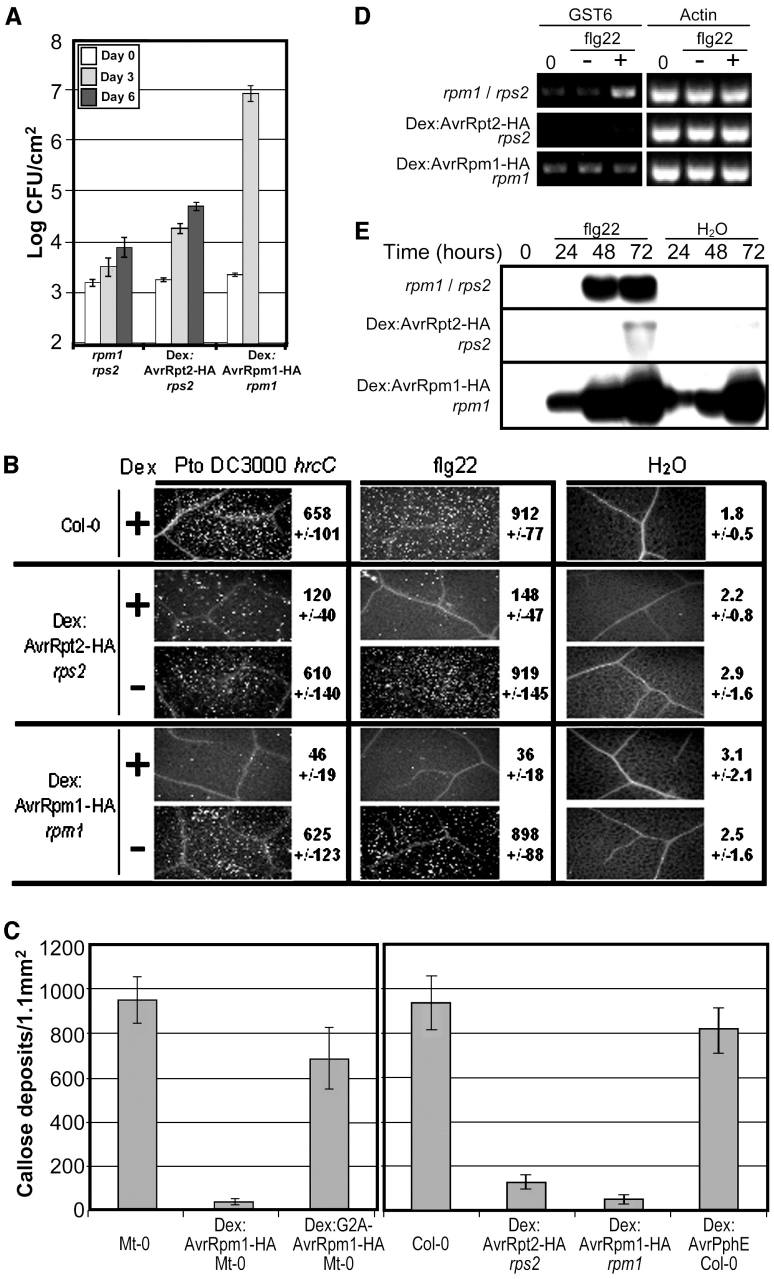

Figure 1. AvrRpt2 and AvrRpm1 Inhibit PAMP-Induced Basal Defense

Plants were treated with Dex 12–18 hr prior to infiltrations.

(A) Growth analysis of Pto DC3000*hrcC* infiltrated at 10^5 cfu/ml. For plants expressing AvrRpm1-HA in *rpm1*, day 6 is missing because the leaves were totally collapsed. Error bars represent the standard deviation from four samples. This experiment is one of three independent replicates.

(B) Aniline blue staining to detect callose deposition. To the right of representative pictures are the average and standard deviation of the number of callose deposits per 1.1 mm^2 from four independent leaves. This experiment is one of five independent replicates. Scale bar is 0.2 mm.

(C) Graphical representation of the number of callose deposits per area as in (B). Each experiment is one of three independent replicates.

(D) RT-PCR detection of *GST6* transcripts. cDNA was prepared from leaves collected at time zero (0) and 1.5 hr after infiltration with water (–) or 35 μM flg22 (+) and subjected to PCR with specific primers for the indicated genes. This experiment is one of three independent replicates.

(E) Western blot to detect PR-1 accumulation. Leaves were collected at the indicated times following infiltration of 10 μM flg22 or water. This experiment is one of six independent replicates.

induced defense signaling. We therefore examined typical defense responses induced by the purified PAMP, flg22, and by Pto DC3000*hrcC* (Figures 1B–1E), including callose deposition, the rapid transcriptional activation of a particular glutathione S-transferase gene, *GST6*, and the late accumulation of pathogenesis related protein 1 (PR-1).

Utilizing transgenic plants expressing AvrRpt2 and AvrRpm1, we found that both significantly inhibited callose deposition induced by TTSS-deficient bacteria and flg22 (Figure 1B). As a control, we determined that an inactive derivative of AvrRpm1 failed to inhibit callose deposition induced by TTSS-deficient bacteria and flg22. This mutant, AvrRpm1(G2A), is not myristoylated like its wild-type counterpart and therefore not properly localized inside plant cells (Nimchuk et al., 2000). Both the AvrRpm1(G2A) mutant and wild-type AvrRpm1 were analyzed in the *RPM1* null ecotype Mt-0 (Figure 1C). Interestingly, an unrelated type III effector, AvrPphE, did not reduce flg22-induced callose in Col-0, which lacks an R protein specific to AvrPphE (Figure 1C). Thus, while both AvrRpt2 and AvrRpm1 both block callose deposition, this is not a universal characteristic of type III effectors.

AvrRpt2 and AvrRpm1 also both inhibited flg22-induced activation of *GST6* transcription (Figure 1D). AvrRpt2 blocked the accumulation of PR-1 induced by flg22 (Figure 1E). Expression of AvrRpm1 induced high-

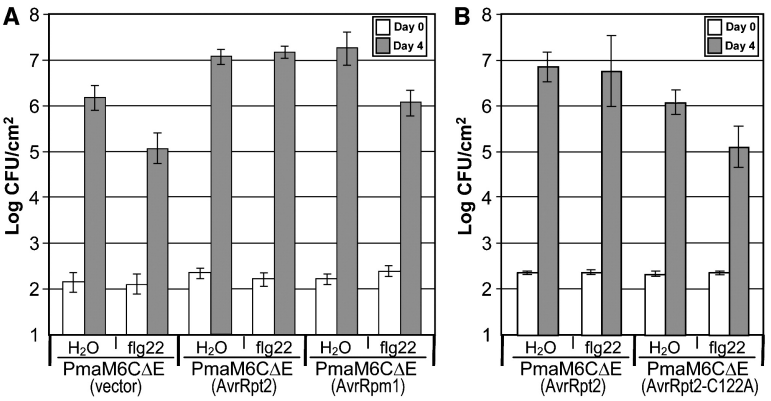

Figure 2. AvrRpt2 Overcomes PAMP-Induced Bacterial Growth Repression

Growth analysis of Pma M6CΔE coinfiltrated at 10^4 cfu/ml with either water or 10 μM flg22 in *rpm1/rps2* plants.

(A) Test of bacteria carrying empty vector or a plasmid expressing AvrRpt2 or AvrRpm1. This experiment is one of seven independent replicates.

(B) Test of bacteria carrying a plasmid expressing AvrRpt2 or a protease-deficient derivative of AvrRpt2 (C122A). This experiment is one of three independent replicates.

level accumulation of PR-1 (Figure 1E) that precluded any conclusions about its effect on flg22-induced PR-1 expression. Thus, AvrRpm1 inhibited two, and AvrRpt2 inhibited all three of these PAMP-induced defense readouts. Surprisingly, AvrRpt2 weakly and AvrRpm1 strongly induced PR-1 accumulation independent of bacteria or purified PAMP (water controls, Figure 1E). This may result from additional activities of these type III effector proteins (see Discussion).

AvrRpt2 Inhibits PAMP-Induced Growth Repression of Virulent Bacteria

When infiltrated into the leaves of *Arabidopsis*, flg22 can inhibit the growth of virulent gram-negative bacteria (Zipfel et al., 2004). We used this assay to test the ability of AvrRpt2 and AvrRpm1, when delivered by bacteria, to overcome PAMP-induced growth suppression of the bacteria (Figure 2). In these experiments, the type III effectors were delivered via type III secretion from bacteria rather than being expressed directly in the plant. We used *P. syringae* pv. *maculicola* M6 CΔE (Pma M6CΔE), a strain whose weak virulence can be complemented with AvrRpm1 (Rohmer et al., 2003) or with AvrRpt2 (Figure 2A). When Pma M6CΔE carrying empty plasmid was coinfiltrated with flg22, bacterial growth was inhibited compared to infiltration of bacteria alone. When bacteria expressing AvrRpt2 were coinfiltrated with flg22, their growth was unchanged relative to when they were infiltrated alone. Thus, AvrRpt2 inhibits flg22-induced growth repression. When bacteria expressing AvrRpm1 were coinfiltrated with flg22, they grew less than when they were infiltrated alone but better than bacteria carrying empty plasmid that were coinfiltrated with flg22. Thus, AvrRpm1 may be incompletely inhibiting flg22-induced growth repression. Alternatively, AvrRpm1 may have an additional activity independent of its ability to inhibit flg22-induced growth repression (see Discussion).

We determined the contribution of the AvrRpt2 protease activity to its inhibition flg22-induced growth repression. We tested a protease-inactive derivative of AvrRpt2 (C122A) in which a cysteine at the probable catalytic site was changed to alanine (Axtell et al., 2003). When infiltrated with water, bacteria expressing this derivative of AvrRpt2 grew to ~10% the level of bacteria expressing wild-type AvrRpt2 (Figure 2B). Furthermore, when the bacteria are coinfiltrated with flg22, the derivative of AvrRpt2 is unable to overcome the induced growth repression (Figure 2B). Thus, protease activity is required for the contribution of AvrRpt2 to fitness of Pma M6CΔE.

Overexpression of RIN4 Inhibits PAMP-Induced Defense Signaling

Because AvrRpt2 and AvrRpm1 both inhibit PAMP-induced defense responses and both target RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2002; Mackey et al., 2003), we investigated the contribution of RIN4 to PAMP signaling. We tested the growth of Pto DC3000*hrcC* on plants that conditionally overexpress RIN4 in the wild-type Col-0 background (Dex:RIN4, Figure 3A). Pto DC3000*hrcC* grew 10-fold over 6 days in Col-0 plants. The same bacteria grew over 1000-fold on two independent homozygous lines of Dex:RIN4 following Dex treatment. The amount of RIN4 in these lines prior to Dex treatment is similar to that in Col-0 plants and increases significantly following Dex treatment (Figure 3B).

We also tested whether overexpression of RIN4 inhibits specific PAMP-induced defense responses. Overexpression of RIN4 in Dex:RIN4 inhibited callose deposition induced by TTSS-deficient bacteria or by flg22 (Figure 3C) and expression of *GST6* induced by flg22 (Figure 3D). The inhibition of these PAMP-induced responses parallels that seen in plants expressing AvrRpt2 and AvrRpm1 (Figures 1B and 1D). Overexpression of RIN4 also induces strong, PAMP-independent expression of PR-1 (data not shown), similar to conditional expression of AvrRpm1 in transgenic plants (Figure 1E). Thus, similar to AvrRpt2 and AvrRpm1, overexpression of RIN4 inhibits defense readouts induced by PAMP signaling.

The Absence of RIN4 Enhances PAMP-Induced Defense Signaling

The inhibition of a process by overexpression of a protein does not necessarily demonstrate the normal function of that protein in the process. Therefore, we tested PAMP-induced responses in plants lacking RIN4. Consistent with the hypothesis that RIN4 is a negative regulator of PAMP signaling, these plants displayed en-

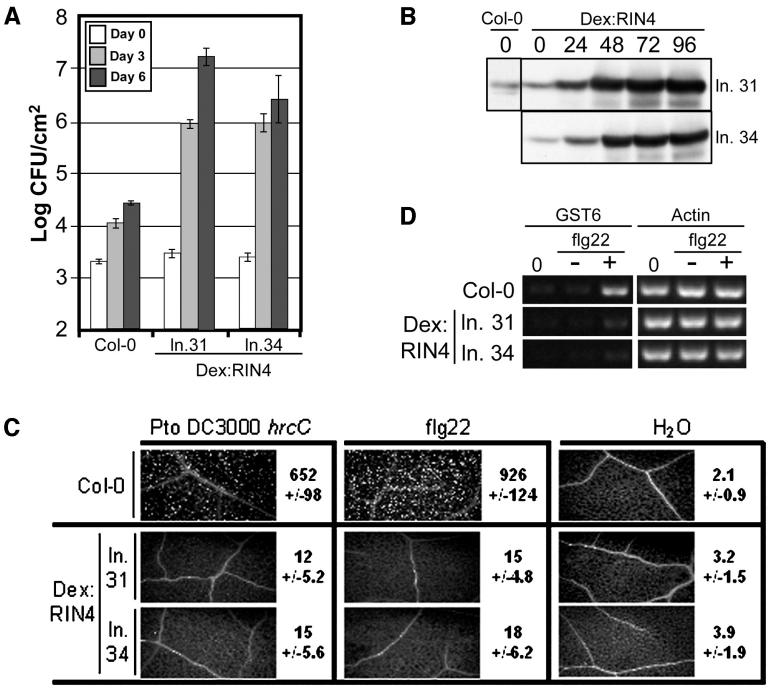

Figure 3. Overexpression of RIN4 Inhibits PAMP-Induced Basal Defense

Col-0 and two independent Dex:RIN4 lines were treated with Dex 24 (for [A]) or 48 (for [C] and [D]) hours prior to infiltrations.

(A) Growth analysis of *Pto* DC3000*hrcC* as in Figure 1. This experiment is one of four independent replicates.

(B) Western blot showing levels of RIN4 protein in Col-0 and in the Dex:RIN4 lines. Samples were collected from unsprayed plants (0) and at the indicated number of hours after spraying with Dex.

(C) Aniline blue staining of callose as in Figure 1. This experiment is one of four independent replicates.

(D) RT-PCR detection of *GST6* transcripts as in Figure 1. This experiment is one of three independent replicates.

hanced PAMP-induced responses relative to plants expressing RIN4 (Figure 4).

We examined PAMP-induced callose deposition (Figure 4A). Using the concentration of flg22 from our standard experiments we noticed a bias toward more callose in plants lacking RIN4 (*rpm1/rps2/rin4*) than in control plants (*rpm1/rps2*). When lower concentrations of flg22 were tested, earlier and stronger accumulation

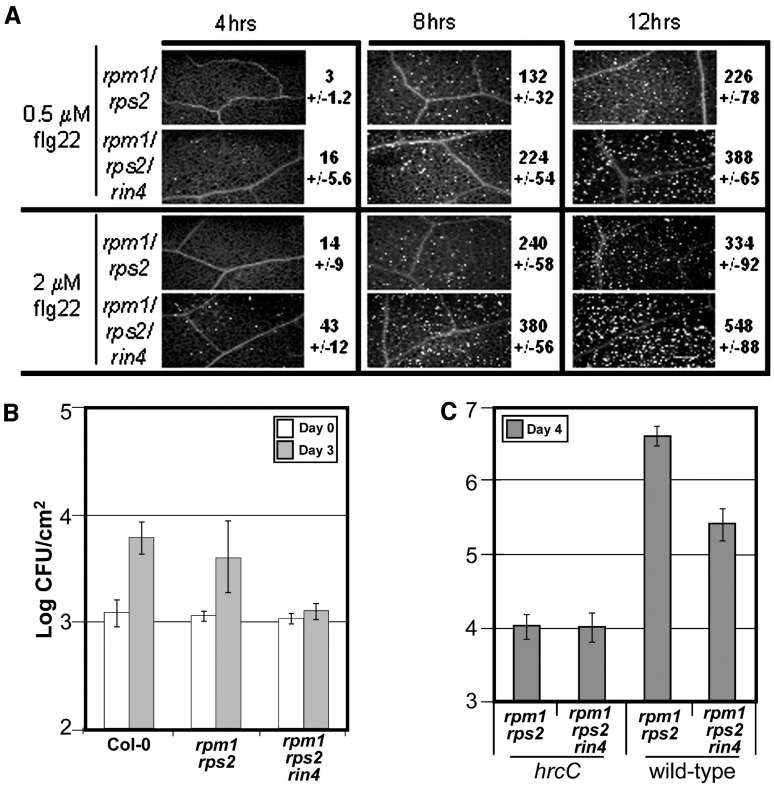

Figure 4. The Absence of RIN4 Enhances PAMP-Induced Basal Defense

rpm1/rps2 and *rpm1/rps2/rin4* plants were compared.

(A) Aniline blue staining of callose following inoculation with low concentrations of flg22. This experiment is one of three independent replicates.

(B) Growth analysis of Pto DC3000*hrcC* as in Figure 1. This experiment is representative of five out of six independent replicates.

(C) Growth analysis of Pto DC3000*hrcC* and Pto DC3000 following spray inoculation. This experiment is one of six independent replicates.

of callose in plants lacking RIN4 was apparent. Similarly, flg22 tended to induce PR-1 expression more quickly and strongly in plants lacking RIN4 (data not shown). Thus, RIN4 negatively regulates flg22-induced defense responses.

We hypothesized that enhanced defense signaling in plants lacking RIN4 might correlate with enhanced resistance to bacteria. The growth of Pto DC3000*hrcC* was significantly reduced in plants lacking RIN4 (Figure 4B), indicating a more effective defense. Thus, RIN4 negatively regulates a defense that is effective against TTSS-deficient bacteria.

We also tested whether the enhanced defenses in plants lacking RIN4 could affect the growth of wild-type bacteria. We recently showed that wild-type Pto DC3000 grows equally well when infiltrated into the leaves of *rpm1/rps2* and *rpm1/rps2/rin4* (Belkhadir et al., 2004). Similarly, Pto DC3000 grows equally well when infiltrated into the leaves of *FLS2* and *fls2* plants (Zipfel et al., 2004). However, a contribution of FLS2 to an effective defense against Pto DC3000 is apparent when the bacteria are inoculated via spraying onto the surface of leaves (Zipfel et al., 2004). Because bacterial numbers are measured after surface sterilization of the leaves, this experiment measures colonization of the interior of the leaf as well as growth. This protocol contrasts with inoculation experiments that measure only growth following physical introduction of bacteria into the interior of the leaf. Because our results (Figures 3C, 3D, and 4A) indicate that RIN4 regulates function of FLS2, we used this assay to examine the effect of RIN4 on bacterial growth (Figure 4C). Following spray inoculation, levels of Pto DC3000*hrcC* were low and unaffected by the status of RIN4; these TTSS-deficient bacteria inefficiently colonized the leaf. On the contrary, wild-type Pto DC3000 successfully colonized *rpm1/rps2* plants. Importantly, the ability of these wild-type bacteria to colonize *rpm1/rps2/rin4* plants was significantly reduced. Thus, RIN4 negatively regulates a defense that limits the ability of Pto DC3000 to colonize the leaf.

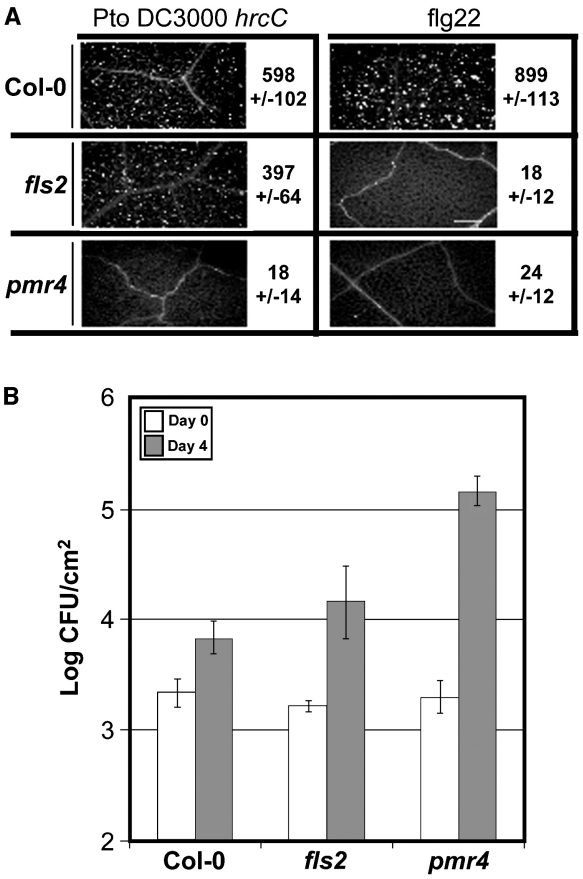

Figure 5. PMR4-Dependent Callose Deposition Contributes to Growth Suppression of TTSS-Deficient Bacteria

Col-0, *fls2*, and *pmr4* were compared.

(A) Aniline blue staining of callose as in Figure 1. This experiment is one of three independent replicates.

(B) Growth analysis of Pto DC3000*hrcC* as in Figure 1. This experiment is one of three independent replicates.

FLS2 and Other Receptors Induce PMR4-Dependent Callose Deposition

We hypothesized that TTSS-deficient Pto DC3000*hrcC* display multiple PAMPs. To test the relative contribution that flagellin plays in the defense response to Pto DC3000*hrcC*, we tested plants with a T-DNA insertion that disrupts the promoter of *FLS2* (Zipfel et al., 2004). As expected, FLS2 is required for flg22-induced callose deposition. However, callose deposition induced by TTSS-deficient bacteria was only partially reduced (~30%) in the *fls2* background (Figure 5A). Thus, FLS2 makes a quantitative contribution to the overall signal induced by TTSS-deficient bacteria that leads to the deposition of callose.

We also assessed the contribution of PMR4 to callose deposition induced by both flg22 and by TTSS-deficient bacteria. PMR4 is the callose synthase required for callose deposition in response to the fungal pathogens *Erysiphe* and *Blumeria* (Jacobs et al., 2003; Nishimura et al., 2003). Neither TTSS-deficient bacteria nor flg22 induced callose in *pmr4*. Thus, FLS2 and another receptor(s) engaged by TTSS-deficient bacteria induce signals that converge and cause PMR4-dependent callose deposition.

PMR4 Contributes to PAMP-Induced Defense

We tested the relative contributions of FLS2 and PMR4 to defense against TTSS-deficient bacteria. The growth of Pto DC3000*hrcC* was measured following infiltration into *fls2* and *pmr4* plants (Figure 5B). In all three experiments, Pto DC3000*hrcC* grew slightly better on *fls2* than on the wild-type plants, but in no experiment was this difference statistically significant. In *pmr4*, the TTSS-deficient bacteria increased their numbers 20-fold more than in the wild-type plants. PMR4 therefore makes a contribution to the defense response against Pto DC3000*hrcC*. Taken together, the data in Figure 5 indicate that the plant uses FLS2 and other receptors to induce a basal defense response that requires PMR4-dependent callose deposition to be fully effective.

Discussion

We demonstrate that the type III effector proteins AvrRpt2 and AvrRpm1 inhibit PAMP-induced defense

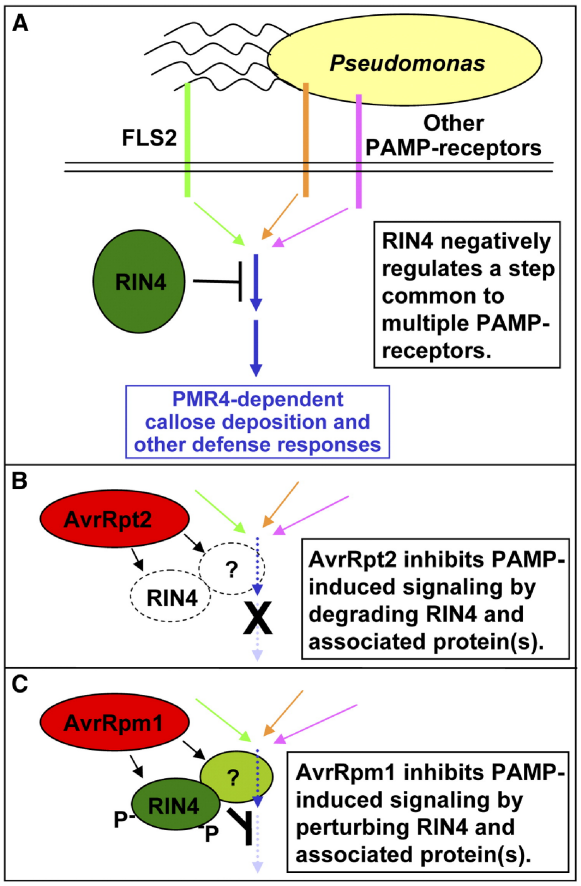

Figure 6. Model: AvrRpt2 and AvrRpm1 Inhibit PAMP-Induced Defense Responses by Manipulating RIN4 and Associated Protein(s)

(A) Bacteria are recognized by FLS2 (green) and other PAMP receptors (orange and purple). RIN4 negatively regulates the induced signaling.

(B) AvrRpt2 destroys RIN4 and associated protein(s).

(C) AvrRpm1 perturbs RIN4 and associated protein(s).

responses. RIN4, a target of these type III effectors, negatively regulates PAMP signaling. PAMP-induced defense responses are inhibited or enhanced in plants overexpressing or lacking RIN4, respectively. Regulation of PAMP-induced defenses is correlated with the ability of bacteria to proliferate in planta. Expression of AvrRpt2 or AvrRpm1 or overexpression of RIN4 inhibits both FLS2-dependent and FLS2-independent defense responses. We therefore propose a model in which RIN4 negatively regulates PAMP-driven signal transduction downstream of FLS2 and additional, putative PAMP receptors (Figure 6). We suggest that AvrRpt2 and AvrRpm1 inhibit PAMP-induced signaling via manipulation of RIN4 and associated proteins. PAMP signaling induces PMR4-dependent callose deposition, which we establish as a component of an effective defense response against TTSS-deficient *P. syringae*. Our results establish a framework in which to study how these type III effectors disrupt PAMP-induced defense signaling through the targeting of RIN4 and associated proteins.

This work significantly augments the recently emerging guard hypothesis. It was previously shown that (1) AvrRpt2 and AvrRpm1 distinctly perturb RIN4 and (2) RPS2 and RPM1 transduce the induced perturbations of RIN4 into defense responses that protect the plant against pathogens deploying AvrRpt2 and AvrRpm1, respectively (Axtell and Staskawicz, 2003; Mackey et al., 2002; Mackey et al., 2003). This work led to speculation that targeting of RIN4 by AvrRpm1 and AvrRpt2 contributes to their ability to enhance bacterial virulence. We now show that AvrRpt2 or AvrRpm1 manipulate RIN4 and associated proteins in order to inhibit PAMP-induced defense signaling. Thus, we suggest that RPS2 and RPM1 "guard" the plant against pathogens deploying type III effectors that inhibit PAMP signaling. The generality of these findings is as yet untested, though supporting data are emerging. For example, PBS1 is a proteolytic target of the type III effector, AvrPphB, and RPS5 induces defense responses upon cleavage of PBS1 (Shao et al., 2003). Perhaps cleavage of PBS1 contributes to an as yet unidentified virulence activity of AvrPphB. AvrA from *S. typhimurium* inhibits innate immune signaling in animal cells and has also been shown to induce an apoptotic response reminiscent of the plant HR (Collier-Hyams et al., 2002). Perhaps an animal equivalent to a plant R protein is "guarding" a host target of AvrA.

Regulation of PAMP signaling is likely an evolutionarily ancient function of RIN4. Consider the complex interactions involving PAMP signaling, RIN4, type III effectors, RPS2, and RPM1. At least two type III effector proteins inhibit PAMP signaling by manipulating RIN4 and presumably associated proteins (Belkhadir et al., 2004). Involvement of RIN4 and its associates in PAMP signaling likely predated targeting of this function by type III effector proteins. The evolution of R proteins that perceive type III effector-induced perturbations of RIN4 could not have evolved until after the appearance of type III effectors that induce those perturbations. Thus, we propose that regulation of PAMP signaling is an ancient, perhaps original, function of RIN4. Later, type III effector proteins (and perhaps as yet undiscovered virulence factors from other classes of plant pathogens) evolved that manipulate RIN4 in the process of inhibiting PAMP signaling. Finally, plants evolved RPS2 and RPM1 to "guard" RIN4 and thus detect pathogen-derived perturbations of PAMP-induced signal transduction. Study of the targets of other type III effector proteins should reveal the spectrum of R protein-independent host processes that they manipulate.

RIN4 is a negative regulator of plant defense signaling induced by multiple PAMP receptors and R proteins. A pure PAMP, flg22, induces FLS2- and PMR4-dependent callose deposition. TTSS-deficient bacteria still induce PMR4-dependent callose deposition in *fls2* plants. Thus, it seems likely that *P. syringae* present additional PAMPs, such as LPS (Keshavarzi et al., 2004; Newman et al., 2002), that engage PAMP receptors other than FLS2. Signaling induced by both FLS2 and these putative additional PAMP receptors is blocked by AvrRpt2 or AvrRpm1 or by overexpression of RIN4. Furthermore, PAMP-induced defense signaling is enhanced in the absence of RIN4. RIN4 has no motifs predictive of enzyme function; thus, we envision that it is an adaptor protein involved in negatively regulating signal transduction from multiple PAMP receptors. Function of RIN4 likely occurs at the plasma membrane. Ac-

tivity of AvrRpm1 requires myristoylation-dependent targeting to the plasma membrane (Nimchuk et al., 2000), and AvrRpt2 and RIN4 are also membrane associated (Axtell et al., 2003; Mackey et al., 2003). Thus, it is at the membrane that AvrRpt2 and AvrRpm1 presumably target RIN4. FLS2 is a transmembrane protein with an intracellular kinase domain necessary for its function (Gómez-Gómez and Boller, 2000). We suspect that AvrRpt2 or AvrRpm1 inhibit FLS2 function at the membrane, prior to the activation of MAP-kinases (Asai et al., 2002) and other downstream signaling components. The ability of RIN4 to negatively regulate PAMP signaling is consistent with its known ability to negatively regulate ectopic activation of RPS2 (Mackey et al., 2003) and RPM1 (Belkhadir et al., 2004). RPS2 and RPM1 each interact with RIN4 and, although not predicted to be integral to the membrane, each is also membrane associated (Axtell and Staskawicz, 2003; Boyes et al., 1998; Mackey et al., 2002; Mackey et al., 2003). Thus, we suggest that RIN4, RPS2, RPM1, and components that transduce defense signaling from R proteins and PAMP receptors exist as membrane-associated complexes.

RIN4 is not the only target of AvrRpt2 in the plant (Belkhadir et al., 2004). AvrRpt2 inhibits PAMP signaling despite the fact that it induces disappearance of a negative regulator of PAMP signaling. The protease activity of AvrRpt2 is necessary for suppressing PAMP-induced bacterial growth restriction. We therefore propose that AvrRpt2 proteolytically targets a protein(s) associated with RIN4 that is positively required for PAMP-dependent signal transduction (Figure 6B). AvrRpt2 is expressed as a proenzyme that is cleaved, in the presence of a eukaryotic host factor, into the active form (Jin et al., 2003; Mudgett and Staskawicz, 1999). The amino acid sequence where AvrRpt2 cleaves itself is highly similar to two sites in RIN4 (Jones and Takemoto, 2004). This sequence likely represents a target site for the protease activity of AvrRpt2. This idea was born from the observation that a conserved sequence specifies autocleavage of AvrPphB and cleavage of its target in *Arabidopsis*, PBS1 (Shao et al., 2003). The putative target site of AvrRpt2 may be present in an additional substrate(s). For example, members of a small family of RIN4-related proteins contain one or two of the putative cleavage sites from within RIN4. It will be interesting to determine whether AvrRpt2 inhibits PAMP signaling by proteolytically degrading any of these proteins.

In plants, PAMP receptors and R proteins signal through common proteins, including RIN4 (Jones and Takemoto, 2004). RNA profiling experiments demonstrated significant overlap between the transcriptional responses induced by PAMP receptors and R proteins (Maleck et al., 2000; Navarro et al., 2004; Tao et al., 2003). Thus, when an R protein recognizes a defense-suppressing type III effector protein, it presumably hyperactivates either the same or a highly interdigitated signal transduction pathway that was targeted by the type III effector. This is intuitive, since an R protein that is monitoring the integrity of a defense signaling pathway would presumably be in the correct subcellular location to efficiently activate that same pathway. Germane to this idea is the observation that plant defense responses to NPP1, a PAMP from *Phytophthora*, require PAD4 and NDR1 (Fellbrich et al., 2002). PAD4 and NDR1 are also required for the function of numerous R proteins. In particular, NDR1 is required for normal function of RPS2 and RPM1 (Century et al., 1995). Based on these findings, we predict that AvrRpt2 and AvrRpm1 will also inhibit plant defense responses against NPP1. Testing the ability of type III effectors to inhibit signaling induced by various PAMPs will provide valuable insight into both type III effector function and defense signal transduction.

Classical markers of defense may not always be indicative of an effective defense response by the plant. Responses that are effective against one pathogen can be ineffective or even counterproductive against another pathogen (Govrin and Levine, 2000; Kunkel and Brooks, 2002). We show that the growth of TTSS-deficient bacteria is significantly enhanced by expression of AvrRpm1 and overexpression of RIN4, which strongly induce expression of PR-1. Similar, apparently contradictory results were obtained for the role of PMR4-dependent callose deposition in combating *Erysiphe* and *Blumeria*. In these cases, the absence of callose in *pmr4* is associated with enhanced resistance against these fungal pathogens. Thus, callose either hampers antifungal defenses of the plant or it is a required factor for fungal colonization. In contrast, PMR4-induced callose deposition constitutes part of an effective defense response against TTSS-deficient bacteria and possibly also against wild-type bacteria. It may be a common phenomenon that plants induce complex defense responses of which a subset is disadvantageous in combating a particular pathogen. The induction of PR-1 by AvrRpm1 may reveal an added layer of complexity; virulence factors might actively promote ineffective or beneficial "defense" responses of the plant.

AvrRpt2 and AvrRpm1 enhance bacterial growth in plants lacking RIN4 (Belkhadir et al., 2004). This observation fits with our results in either of two ways. First, the ability of these effectors to inhibit PAMP signaling may not require the presence of RIN4. The inhibition could result from AvrRpt2-dependent proteolysis (Figure 6B) or AvrRpm1-dependent perturbation (Figure 6C) of RIN4-associated proteins. Consistent with this idea, AvrRpm1 can inhibit PAMP-induced callose deposition in plants lacking RIN4 (M.G.K. and D.M., unpublished data). A second, nonexclusive possibility is that AvrRpt2 and AvrRpm1 have additional virulence functions, independent of the ability to inhibit PAMP signaling. The data presented here is consistent with the notion that AvrRpm1 has such an additional activity. Expression of AvrRpt2 and AvrRpm1 each inhibit PAMP signaling in response to TTSS-deficient bacteria. Nonetheless, AvrRpm1 is significantly better at enhancing the growth of these bacteria. The identification of targets, other than RIN4, that are perturbed by AvrRpt2 and AvrRpm1 will shed further light on how bacterial type III effector proteins perturb host processes to promote pathogen virulence.

Experimental Procedures

Plants

All the plants used in this work were in the wild-type Col-0 background. The mutants used are as follows: *rps2-101C* has a stop

codon at amino acid 235 of *RPS2*; *rpm1-3* has a stop codon at amino acid 87 of *RPM1*; *rin4* has a T-DNA insertion after amino acid 146 of *RIN4*; *pmr4-1* has a stop codon at amino acid 687 of *PMR4* (also known as *GSL5* and *CalS12*) (Nishimura et al., 2003); *fls2* has a T-DNA insertion in the promoter of *FLS2* that abolishes expression of the gene (Zipfel et al., 2004). The *rpm1/rps2/rin4* triple mutant was constructed by marker-assisted breeding (Belkhadir et al., 2004).

Transgenic Plants

To conditionally overexpress RIN4, the wild-type gene was cloned into pTA7002 (Aoyama and Chua, 1997). Transgenic plants were generated by vacuum infiltrating *Agrobacterium tumerifaciens* (GV3101) carrying this plasmid into flowering Col-0. Transgenic progeny were selected by growth on plates of Gamborg's B5 (Gibco) with 20 μM Hygromycin B (Sigma). Independent lines with single insertion loci were identified and propagated to homozygosity. Col-0 plants inducibly expressing AvrRpt2-HA and AvrRpm1-HA are in *rps2* and *rpm1* backgrounds, respectively (Mackey et al., 2003; Mackey et al., 2002). Mt-0 plants inducibly expressing AvrRpm1-HA and G2A-AvrRpm1-HA were described previously (Nimchuk et al., 2000). Col-0 plants inducibly expressing AvrPphE were a kind gift of S.Y. He. All lines conditionally expressing bacterial effectors were generated using pTA7002. Expression of RIN4 or the effectors was induced by spraying with 20 μM Dexamethasone (Sigma-Aldrich) containing 0.005% silwet L-77 (CKWitco Corporation).

Bacteria

The TTSS-deficient bacteria used in this report are *P. syringae* pv. *tomato* (Pto) strain DC3000 containing a mutation in *hrcC*. The virulent bacteria used in this report are *P. syringae* pv. *tomato* (Pto) strain DC3000 and the *P. syringae* pv. *maculicola* strain M6CΔE (Rohmer et al., 2003) harboring empty vector (pVSP61) or derivatives of this plasmid expressing *avrRpm1* or *avrRpt2*. In Figure 2B, Pma M6CΔE carry wild-type or mutant AvrRpt2 on plasmid pDSK519n (Axtell et al., 2003).

Growth curves (except Figure 4C) were conducted by inoculating bacterial suspensions in 10 mM $MgCl_2$ into leaves of five-week-old plants with a needleless 1 ml syringe. Pto DC3000*hrcC* was infiltrated at 10^5 cfu/ml. After the infiltrated leaves were dry (about 4 hr), the plants were kept in 100% humidity (clear dome on) for the remainder of the experiment. Pma M6CΔE was infiltrated at 10^4 cfu/ml and was coinfiltrated with water or 10 μM flg22. These plants remained uncovered for the remainder of the experiment. Growth analysis of Pto DC3000 was conducted as described in Zipfel et al. (2004). Briefly, bacteria were resuspended at 5×10^8 in 0.04% silwet and sprayed onto the surface of six-week-old plants. Plants were kept covered for 4 hr, and then the dome was removed for the remainder of the experiment. After 4 days, leaf discs were collected and surface-sterilized in 70% ethanol for 30 s. For all growth experiments, leaf discs were ground to homogeneity in 10 mM $MgCl_2$ and the titer determined by serial dilution and plating.

Protein

Approximately 3 cm^2 of leaf tissue was ground in 100 μl of grinding buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM DTT, and plant protease inhibitor cocktail [Sigma-Aldrich]) and insoluble debris was pelleted by centrifugation at 20,000 × g for 10 min at 4°C. Protein concentration of the soluble supernatant was determined by the Bio-Rad protein assay (Bio-Rad). Samples were resolved on SDS-PAGE gels (mini protean, Bio-Rad) of 12% and transferred to PVDF membrane (Millipore). Western blots were done by standard methods. Anti-RIN4 sera (Mackey et al., 2002) and anti-PR-1 sera (Kliebenstein et al., 1999) were used at dilutions of 1:5,000 and 1:10,000, respectively.

RNA

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 4 μg of total RNA by using universal oligo(dT) primer and the ThermoScript reverse transcriptase from the ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's instructions. PCR amplifications were carried out with 0.05% of the cDNA product using primers (5′-CAGTATCAAGGTTCACGGAGTTC CCATG-3′ and 5′-AGGCAAGCTTAGAGGCGTTAG GGTCAA-3′) for *GST6* (At2g47730) or (5′-CTAAGCTCTCAAGATCAAAGGCTTA-3′ and 5′-TTAACATTGCAAAGAGTTTCAAGGT-3′) for *actin2*.

Callose Staining

Four-week-old leaves were syringe-infiltrated with 10^8 cfu/ml of Pto DC3000*hrcC*, 100 μM flg22 (or as indicated in Figure 4), or distilled water and collected after 15 hr (or as indicated in Figure 4). Whole leaves were collected, stained with Aniline blue (Hauck et al., 2003), mounted in 50% glycerol, and examined with epifluorescent illumination from a Nikon microscope. Four leaves were prepared for each treatment. Representative views of these pictures were randomized, and the number of callose deposits was counted blind.

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