

Interference between Two Specific Pathogen Recognition Events Mediated by Distinct Plant Disease Resistance Genes

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We demonstrate that the interaction of the avirulence gene *avrRpt2* and the cognate resistance gene *RPS2* interferes with the interaction of *avrRpm1*–*RPM1* in *Arabidopsis*. Interference is mediated outside of the bacterial pathogen *Pseudomonas syringae*, presumably at the level of recognition of *avr*-dependent signals, yet does not require the wild-type *RPS2* product. A numerical excess of *P. syringae* expressing *avrRpm1* can overcome this interference in mixed inoculations. The interference of *avrRpt2*–*RPS2* engagement with *RPM1*-dependent functions is mirrored by transcriptional activation of genes preferentially expressed during *RPM1*- or *RPS2*-mediated disease resistance reactions. This demonstration of interference between two plant disease resistance genes suggests that their products compete for a common element(s) in a signal transduction pathway leading to disease resistance.

INTRODUCTION

Plants recognize pathogens via disease resistance (*R*) genes, which specifically condition recognition of either the direct or indirect product of a corresponding pathogen avirulence (*avr*) gene. At least five *Pseudomonas syringae* *avr* genes are recognized by four unlinked *Arabidopsis* *R* genes (Dangl, 1993; Kunkel, 1995). The combinations analyzed in our experiments are *avrRpt2*–*RPS2* (Whalen et al., 1991; Innes et al., 1993; Kunkel et al., 1993; Yu et al., 1993) and *avrRpm1*–*RPM1* (Debener et al., 1991; Dangl et al., 1992; Ritter and Dangl, 1995). The respective *avr* genes have never been observed to be present in the same bacterial strain. Interestingly, both *avrRpm1* and *avrB* (Tamaki et al., 1991) are recognized by the same plant *R* gene, *RPM1*, although they share no sequence similarity (Bisgrove et al., 1994; Grant et al., 1995). Both *RPM1* and *RPS2* have recently been cloned and shown to encode related novel proteins containing a putative leucine zipper, a nucleotide binding site, and 14 imperfect leucine-rich repeats of ~24 amino acids (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995). Some of these features are also found in other recently cloned *R* genes (Jones et al., 1994; Whitham et al., 1994; Lawrence et al., 1995; for reviews, see Dangl, 1995; Staskawicz et al., 1995), and this class of *R* genes has been dubbed nucleotide binding site-LRR.

It is not yet known whether these *R* gene products bind their respective *avr*-specific elicitors directly, nor is their subcellular localization directly predictable from the primary amino acid sequence. The number of other plant gene products required for transduction of *R* gene-mediated signals is also unknown, although genetic analyses in several systems have identified a handful of loci required for specific *R* gene function (Freialdenhoven et al., 1994; Hammond-Kosack et al., 1994). In one case, the tomato *Prf* gene, which encodes a nucleotide binding site-LRR protein (J. Salmeron and B. Staskawicz, personal communication), is known to be required for the action of the serine-threonine kinase encoded by the *Pto* gene to determine resistance to strains of *P. syringae* expressing *avrPto* (Martin et al., 1993; Salmeron et al., 1994).

RESULTS

Conjugation of plasmids carrying either *avrRpm1* or *avrRpt2* into either *P. syringae* DC3000 or *P. syringae* Psm M4 (both of which cause disease on all tested *Arabidopsis* accessions; Debener et al., 1991; Whalen et al., 1991) results in strains triggering an idiosyncratic hypersensitive response (HR) on the *Arabidopsis* accession Columbia (Col-0; genotype *RPM1/RPS2*; Table 1). The combination *avrRpm1*–*RPM1* resulted in a visible HR at 5 hr postinoculation, whereas the combination *avrRpt2*–*RPS2* resulted in a weaker HR at ~20 hr

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Table 1. *avrRpt2* Action Interferes with *avrRpm1* Action in Generation of a Hypersensitive Resistance Response

HR Timing on ^a	Pathogenic <i>P. syringae</i> Strain (DC3000 or Psm M4) Expressing					
	No <i>avr</i> Gene	<i>avrRpm1</i>	<i>avrRpt2</i>	<i>avrRpm1</i> + <i>avrRpt2</i>	<i>avrRpm1</i> + <i>avrRpt2::Ω</i>	Mix ^b
<i>RPM1/RPS2</i>	–	+ 5 hr	+ 21 hr	+ 21 hr	+ 5 hr	+ 21 hr
<i>RPM1/rps2</i>	–	+ 5 hr	–	–	+ 5 hr	–
<i>rpm1^{null}/RPS2^c</i>	–	–	+ 6 hr	+ 6 hr	–	+ 6 hr

Bacteria were prepared and inoculated as described by Ritter and Dangl (1995) at an initial inoculum density of 5×10^7 colony-forming units (cfu)/mL. Tissue collapse indicative of HR was monitored visually. Each combination of *P. syringae* and *Arabidopsis* was tested in more than six experiments on a minimum of four individual plants (16 to 20 total leaves) per experiment. Data for expressing both *avr* genes simultaneously are from experiments using pCR105. A minus (–) indicates lack of recognition; a plus (+) followed by timing in hours (hr) indicates HR on all leaves assayed.

^a Plant genotypes are described in the text.

^b Results from mixed inoculations of equal numbers of *P. syringae* expressing either *avr* gene alone. Results for phenotypic HR and plant defense gene induction in mix inoculations with titration are displayed in Figures 3 to 5.

^c Accession Nd-0.

postinoculation. We examined these two sets of specific interactions simultaneously by constructing *P. syringae* strains that carried both *avrRpm1* and *avrRpt2* cloned either into the same replicon or into two compatible replicons (Ritter and Dangl, 1995; see Methods) or by performing mixed inoculations of *P. syringae* expressing either *avrRpm1* or *avrRpt2* (again both DC3000 and Psm M4 transconjugants were tested). We expected that each *avr-R* gene combination would act independently, the rapid HR of accession Col-0 in response to *avrRpm1* would be observed, and the slower response to *avrRpt2* would be masked (Table 1).

In fact, strains expressing both *avr* genes triggered HR on either accession indicative only of the *avrRpt2-RPS2* interaction, including the slower reaction on Col-0, in repeated experiments (Table 1). These results initially suggested that expression of wild-type *avrRpt2* and, potentially, functional engagement of *RPS2* interfered with the *avrRpm1-RPM1* interaction. Several trivial explanations for this observation were ruled out by testing vector and copy number effects in four ways. First, we recovered nearly identical bacterial counts from leaves inoculated with *P. syringae* carrying both *avr* genes on separate vectors (plating for different antibiotic resistance markers present on either *avr* gene vector). These samples were harvested at 5 hr postinoculation, which is the expected timing for an HR caused by the *avrRpm1-RPM1* combination. These titrations (data not shown) demonstrated that the bacteria expressing *avrRpt2* did not kill or inhibit growth of those expressing *avrRpm1* and showed that the plasmid expressing *avrRpm1* is not selectively lost from bacteria carrying both replicons. Second, both *avr* genes were cloned into a single vector called pCR105 (Ritter and Dangl, 1995; see Methods), and the interference of the *avrRpt2-RPS2* combination with *avrRpm1-RPM1* was still observed. Third, a compatible replicon carrying *avrRpt2* with its open reading frame disrupted by insertion of the *Th5Ω* (Whalen et al., 1991) was introduced into *P. syringae* DC3000 carrying *avrRpm1*. In this case, tim-

ing of the HR indicative of the *avrRpm1-RPM1* interaction was restored. Fourth, we used RNA gel blot analysis to show that expression of *avrRpt2* did not influence expression of *avrRpm1*. We grew *P. syringae* DC3000 carrying these *avr* genes either singly or in combination in bacterial culture conditions known to induce *avr* gene transcription, prepared RNA gel blots, and observed no significant difference in transcript levels (data not shown; see Methods).

Table 1 shows the results of inoculation experiments assessing timing of the HR. The *rps2-201* mutant (a point mutation leading to an amino acid exchange in the LRR region; Bent et al., 1994) did not recognize *P. syringae* expressing both *avr* genes, even though *RPM1* is functional in these plants. More importantly, mixed inoculations of *P. syringae* expressing either *avrRpm1* or *avrRpt2* singly (in equal numbers) onto either wild-type Col-0 or *rps2* plants also did not trigger the rapid HR indicative of the *avrRpm1-RPM1* interaction. This result demonstrated that the observed interference is manifested outside the bacteria. Increasing the ratio of bacteria expressing *avrRpm1* in the mixed inoculations, however, restored the rapid *RPM1*-mediated HR (detailed below).

In the absence of recognition, *P. syringae* DC3000 expressing both *avr* genes was capable of sustained growth in leaves of *rps2* plants, although these plants still expressed a functional *RPM1* gene. These analyses are presented in Figure 1 for inoculations of all strains onto leaves of either wild-type Col-0 (*RPM1/RPS2*), a Col-0-derived *rps2* mutant, and the naturally occurring *rpm1^{null}/RPS2* accession Nd-0. As expected, each strain carrying a functional *avr* gene was incapable of sustained growth on wild-type Col-0 (Figure 1A). After inoculation into leaves of the *rps2* mutant, the strain expressing only *avrRpt2* grew, due to the *rps2* mutation (Figure 1B). Importantly, the strain expressing both *avrRpt2* and *avrRpm1* (either cloned together on the pCR105 vector or present on compatible replicons) also grew on the *rps2* mutant. The growth of each strain in the accession Nd-0 (Figure 1C) served as a specificity con-

tol for each gene-for-gene interaction. We also observed this interference when a strain expressing *avrB* was substituted for those expressing *avrRpm1* in mixed inocula (data not shown). Together, data presented in Table 1 and Figure 1 strongly suggest that an *avrRpt2*-dependent signal, acting outside the bacterial pathogen, precludes the triggering of resistance responses indicative of the *avrRpm1*-*RPM1* combination. Moreover, this interference does not require a wild-type *RPS2* product.

We sought further evidence for functional interference of the *avrRpt2*-*RPS2* pathway over that determined by *avrRpm1*-*RPM1* by analyzing *R* gene-mediated, induced expression of plant defense genes. The *ELI3* gene encodes a protein highly related, but not identical, to cinnamyl alcohol dehydrogenases. Its transcription is rapidly induced in an *RPM1*-dependent manner and is not induced in *RPS2*-mediated interactions (Kiedrowski et al., 1992). We harvested total RNA following mixed inoculations of either Col-0 or *rps2* mutant plants at 4 hr postinoculation. We also titrated increasing quantities of *P. syringae* expressing only *avrRpm1* into the mixed inoculum to determine whether both the typical *RPM1* HR at

5 hr and *RPM1*-mediated *ELI3* expression could be restored. The RNA blot analysis displayed in Figure 2 demonstrates several points. First, relatively low levels of *P. syringae* expressing *avrRpm1* are sufficient for both rapid HR at 5 hr and maximal *ELI3* expression. Second, a mixed inoculum with a 2.5-fold input excess of *P. syringae* expressing *avrRpm1* can restore both the rapid *RPM1*-mediated HR and maximal *ELI3* expression. This ratio is apparently at the threshold for such restoration, because in this particular experiment, *RPM1*-mediated HR and *ELI3* expression are restored only in Col-0 plants (see below). Third, in mixed inoculations in which *avrRpt2*-dependent interference was observed (no *RPM1*-dependent HR at 5 hr), only background levels of *ELI3* expression were found in either wild-type Col-0 or *rps2* mutant plants. Fourth, a wild-type *RPS2* protein was not required for interference, as neither the rapid *RPM1*-dependent HR nor induced levels of *ELI3* expression were observed in mixed inocula of an *rps2* mutant. Note that in all cases in which interference was observed, the absolute number of bacteria expressing *avrRpm1* in the mixed inocula was sufficient to trigger maximal *ELI3* induction when inoculated alone.

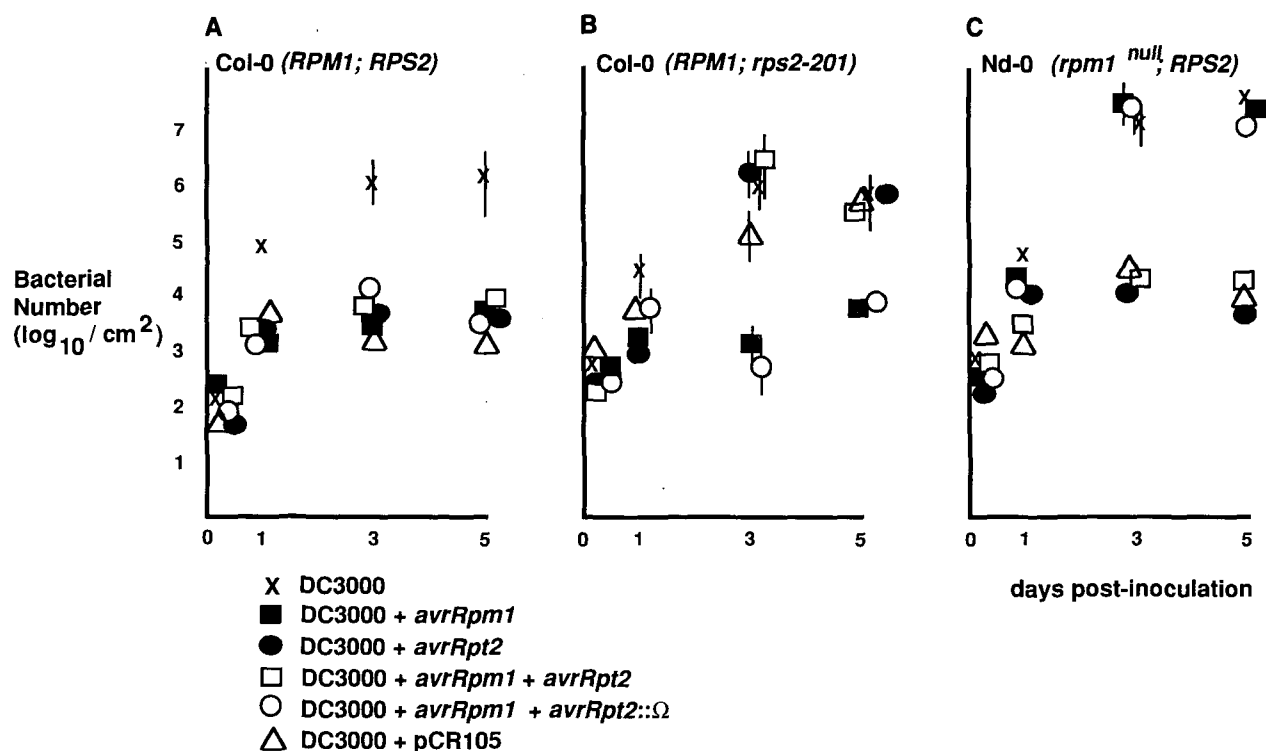


Figure 1. Growth of *P. syringae* DC3000 Carrying either *avrRpm1* or *avrRpt2* Singly, or Both Genes Together, in Arabidopsis Leaves.

(A) to (C) Bacteria were grown and prepared for inoculation, as described by Ritter and Dangl (1995), and inoculated into leaves of adult, 5-week-old Arabidopsis plants (*R* gene compositions are given in [A] to [C]) at an initial concentration of 10^5 cfu/mL. Leaves were harvested at the indicated time points, ground in 10 mM $MgCl_2$, and titrated on media selective for markers on the bacterial chromosome and each vector as described by Ritter and Dangl (1995; see Methods). Each data point represents the mean of three to eight independent experiments, and duplicates of four leaves each were harvested within an experiment. Standard deviation is presented where it was larger than the symbols.

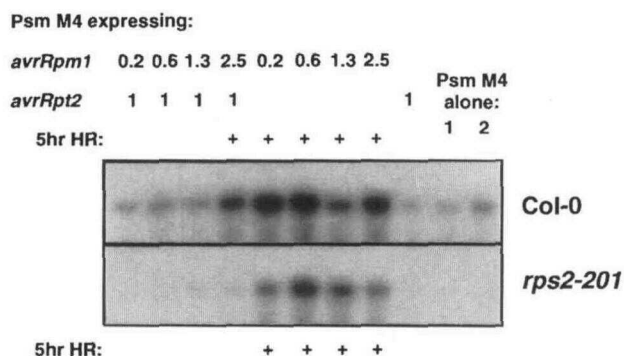


Figure 2. Mixed Inocula Result in Interference with *RPM1*-Dependent Induction of *ELI3* Expression.

Two *P. syringae* Psm M4 transconjugants independently expressing either *avrRpm1* or *avrRpt2* from the pL6 vector were inoculated onto leaves of Col-0 or *rps2-201* plants either alone or after mixing the input amounts indicated (1 represents an initial concentration of 5×10^7 cfu/mL) to achieve various ratios of bacteria expressing each *avr* gene in an inoculum containing at least 5×10^7 /mL total bacteria. Note restoration of the *RPM1*-dependent HR and *ELI3* induction in Col-0 at an input ratio of 2.5:1. RNA was harvested at 4 hr postinoculation and prepared as described in Methods. The probe was an *ELI3* full-length cDNA (Kiedrowski et al., 1992). A (+) indicates visible tissue collapse at 5 hr postinoculation in the remaining inoculated leaves.

In addition to data presented in Figure 2, we performed mixed inocula of wild-type Col-0 and the *rps2* mutant with a second *P. syringae* strain, DC3000, in which *avrRpm1* and *avrRpt2* were expressed from vectors carrying different antibiotic markers on different replicons (pVSP61 for *avrRpm1* and pL6 for *avrRpt2*; see Methods). This allowed determination of absolute numbers of bacterial cells in the leaves at the time of harvest for RNA preparation. In repeated experiments using either *P. syringae* strain, we observed restoration of *RPM1*-dependent HR and *ELI3* induction in Col-0 and the *rps2* mutant at a ratio of $\sim 4:1$ or higher. Maximal levels of *ELI3* expression triggered when delivering *avrRpm1* signal from DC3000 were repeatedly lower than those observed using Psm M4 (data not shown).

We controlled for the occurrence of *RPS2*-mediated signal transduction in mixed inocula in which interference was observed by utilizing the *AIG1* (for *avrRpt2*-induced gene) clone described by Reuber and Ausubel (1996). *AIG1* encodes a novel protein that displays an expression mode opposite to that of *ELI3*; namely, it is induced during *RPS2*-mediated resistance reactions but not during *RPM1*-mediated interactions. The RNA gel blot analysis displayed in Figure 3 demonstrates that *AIG1* expression is observed in those mixed inoculations in which both a rapid *RPM1*-mediated HR and *ELI3* induction were suppressed but an HR at 22 hr postinoculation was visible. Interestingly, inoculation with low absolute doses of bacteria expressing only *avrRpm1* led to some *AIG1* induction in some, but not all, experiments. This induction disappeared as the

absolute dose of *avrRpm1*-expressing bacteria was increased. Figure 3 also demonstrates the specificity of *AIG1* induction, using the accession Nd-0 (genotype *rpm1*^{null}/*RPS2*). We also confirmed that *AIG1* is not induced in *rps2* mutants (data not shown; see Reuber and Ausubel, 1996). Thus, the interference with *avrRpm1*-*RPM1*-dependent signaling we observed is accompanied by induction of *RPS2*-mediated gene induction.

DISCUSSION

Our results clearly demonstrate interference of one gene-for-gene interaction over another. We report two novel findings: first, interference of the *avrRpt2*-*RPS2* interaction with the *avrRpm1*-*RPM1* interaction occurs outside of the two pathogenic *P. syringae* strains used in this study; and second, this interference does not require wild-type *RPS2* protein.

More than 15 *P. syringae* *avr* genes have been cloned over the last decade, and each triggers resistance in appropriate plant lines expressing the corresponding *R* gene (Staskawicz et al., 1984; Dangl, 1994). The role of *avr* genes in initiating *R* gene action remains enigmatic. In addition to *avr* genes, several examples exist of genetically defined inhibitors of *avr* gene action in both phytopathogenic fungi and bacteria (Lawrence et al., 1981; Christ and Groth, 1982; Crute, 1985; Illott et al.,

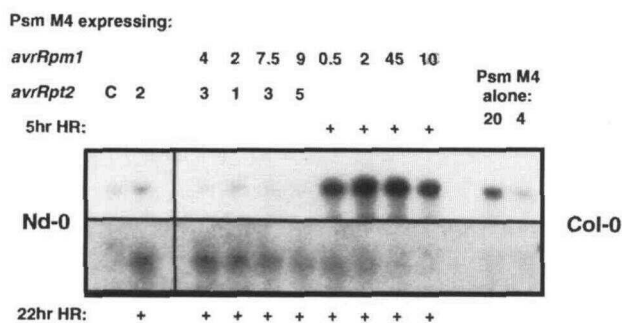


Figure 3. Interference with *avrRpm1*-*RPM1* Signaling Is Accompanied by Induction of *AIG1* Expression.

avr genes were independently expressed in two transconjugants of Psm M4 on different replicons (pVSP61 for *avrRpm1* and pL6 for *avrRpt2*). At 4 hr postinoculation, leaf samples were taken for RNA preparation and for titration. Additional inoculated leaves were observed for an HR at 5 and 22 hr postinoculation. Because the two vectors carry different antibiotic markers, numbers (1 represents 5×10^5 cfu/cm²) reflect titers and ratios of each bacteria in leaves at the time of harvest. The autoradiograph at top is from a blot hybridized with *ELI3* cDNA, as described in the Figure 2 legend. The filter was stripped and reprobbed with the *AIG1* cDNA described by Reuber and Ausubel (1996). RNA from leaves of accession Nd-0 (*R* genotype *rpm1*^{null}/*RPS2*; separated by vertical black line at left) either inoculated with Psm M4 alone (control, labeled C) or expressing *avrRpt2* provides a specificity control for gene induction.

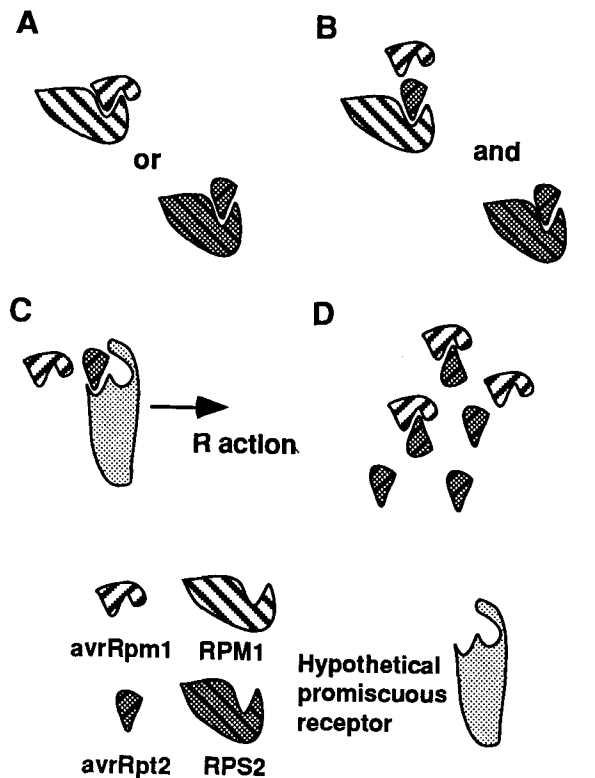


Figure 4. General Hypothetical Models to Explain *R* Gene Interference.

Each symbol is described at the bottom of the figure. See text for details. (A) The independent interaction of each *avr*-derived signal with its cognate *R* gene product.

(B) and (C) Models whereby one *avr*-derived signal competes with the other for binding to either the cognate *R* gene products or a more general promiscuous receptor.

(D) A model of direct interference of one *avr*-derived signal with another.

1989; Lau et al., 1993). One theory is that *P. syringae* *avr* genes are enzymes whose action leads to the biosynthesis of a specific elicitor, which is recognized by the corresponding plant *R* gene. This scenario is supported by analysis of the *avrD* gene, whose product dictates production of a specific elicitor (Keen et al., 1990; Midland et al., 1993). There is no evidence that *avr* gene products are secreted or that any purified *avr*-encoded protein elicits *R* gene-dependent resistance, although this possibility is suggested by analysis of fungal *avr* gene products produced by extracellular pathogens (Van der Ackerveken et al., 1992; Joosten et al., 1994; Rohe et al., 1995). Biochemical characterization of putative *avr* gene-specific elicitors has proven extremely difficult, suggesting that these elicitors are present in vanishingly small quantities or are unstable.

Several general models, outlined in Figure 4, explain our findings that *avrRpt2* is functionally epistatic to *avrRpm1* in a manner independent of wild-type RPS2 protein and occurring outside the bacteria. If the RPM1 and RPS2 proteins do bind their respective *avr*-dependent signals directly, as depicted for

each wild-type interaction in Figure 4A, it may be that the *avrRpt2*-derived signal can also bind to RPM1 in a manner precluding its activity (Figure 4B). This putative interaction could result in shunting of more signal down the RPS2-dependent pathway and could serve to enhance resistance-response specificity. This model is also consistent with our titration data, especially if the binding of the *avrRpt2*-derived signal to RPM1 is of low affinity. In the *rps2* mutant, the *avrRpt2*-derived signal could still bind the RPS2 protein but in a non-productive manner. This model is also consistent with data from Reuber and Ausubel (1996), which show that *R* gene interference can, under some circumstances, work in either direction. (Differences in our observations can most likely be ascribed to slight differences in experimental conditions or vectors employed to deliver *avr* signals, and the sum of our data indicates a fine tuning of these signaling systems.) Alternatively, it could be that the *avr*-dependent signal molecules do not bind to either RPM1 or RPS2 proteins but rather to a common element required for *R* gene action (the hypothetical promiscuous receptor in Figure 4C). Our ability to titrate the observed interference is also consistent with the idea that *avr*-derived signals may compete for a common binding site on a molecule required to trigger *R* gene function. A possible candidate is the gene defined by the *ndr1* mutation, whose action is required for appropriate function of several Arabidopsis *R* genes (Century et al., 1995).

It is also possible that the *avrRpt2*-derived signal directly inhibits action of the *avrRpm1*-derived signal, as shown in Figure 4D. In this case, the putative *avrRpt2* elicitor could bind the *avrRpm1* elicitor, titrating its activity and allowing excess *avrRpt2* elicitor to still act, as depicted in Figure 4D. If the *avr* gene products encode enzymes active outside the bacteria, then this scenario would also be plausible and would suggest that *avrRpt2* is biochemically epistatic to *avrRpm1*. This latter notion is based broadly on biosynthesis of host plant-specific Nod factors. These substituted lipo-oligochitin molecules are required to initiate the series of plant responses required for successful symbiotic colonization of plant roots by various *Rhizobium* species (Dénarié and Cullimore, 1993; Long and Staskawicz, 1993; Schultze et al., 1994). If the *avr*-derived elicitors did share a common structural component, one candidate would be the harpin_{Pss} protein (He et al., 1993; Collmer and Bauer, 1994; Preston et al., 1995), which is sufficient to trigger nonhost HR in several species. This model would then further suggest that *avr* genes encode enzymes that modify or act in concert with the harpin protein outside of the bacterial cell. Because harpin is also a general pathogenicity factor, *avr* gene-modified harpins could also serve as host-specific pathogenicity factors. The recent finding that several *P. syringae* *avr* genes, including *avrRpm1*, are required for maximal pathogenicity supports this idea (Dangl, 1994; Lorang et al., 1994; Ritter and Dangl, 1995).

The availability of cloned *R* genes and *avr* genes in isogenic settings for both host and pathogen and of genes induced in an interaction-specific manner will spur future developments aimed at a molecular resolution of these models.

METHODS

Bacterial Strains and Plasmids

Most bacterial strains and plasmids have been described by Ritter and Dangl (1995), Dangl et al. (1992), and Debener et al. (1991). From Ritter and Dangl (1995), we used pCR106, which is pL6 carrying *avrRpm1*. For these experiments, several new vectors expressing avirulence (*avr*) genes were constructed: pCR107 is pL6 with the HindIII fragment carrying *avrRpt2* from pABL18 (Whalen et al., 1991) cloned into the vector HindIII site. pCR104 carries *avrRpm1* in pLAFR5. We constructed pCR105 from pCR104 by subcloning the same HindIII fragment containing *avrRpt2* into the available HindIII site. pCR105 thus expresses both *avr* genes. A pVSP61 derivative carrying *avrRpm1* was provided by Roger Innes (Indiana University, Bloomington). pLAFR and pL6 derivatives encode tetracycline resistance, and pVSP derivatives encode kanamycin resistance. Transconjugants described in the text were constructed as given by Ritter and Dangl (1995). Expression of each *avr* gene in these transconjugants was controlled by RNA blot analysis, using bacterial culture conditions known to induce *avr* gene expression (Ritter and Dangl, 1995). We used polymerase chain reaction–amplified fragments as probes (Ritter and Dangl, 1995) for RNA blots, which demonstrated that each *avr* gene on pCR106 was expressed in roughly equivalent amounts and that each *avr* gene when carried separately was also expressed.

Plant Care and Inoculations

All inoculations and growth curves were performed as described by Ritter and Dangl (1995).

RNA Gel Blot Analysis

RNA was extracted from leaves inoculated with bacteria or with buffer as a control and prepared and blotted as described by Kiedrowski et al. (1992). The *ELI3* and *AI61* probes were gel-isolated inserts labeled by using a random priming kit (Boehringer Mannheim). An actin probe was used to check for equal loading in all experiments (data not shown). RNA was harvested from six plants per experimental treatment, and blots shown are indicative of two to four independent RNA experiments.

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