The Leucine-Rich Repeat Domain Can Determine Effective Interaction Between
RPS2 and Other Host Factors in Arabidopsis RPS2-Mediated Disease Resistance

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ABSTRACT
Like many other plant disease resistance genes, Arabidopsis thaliana RPS2 encodes a product with nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains. This study explored the hypothesized interaction of RPS2 with other host factors that may be required for perception of Pseudomonas syringae pathogens that express avrRpt2 and/or for the subsequent induction of plant defense responses. Crosses between Arabidopsis ecotypes Col-0 (resistant) and Po-1 (susceptible) revealed segregation of more than one gene that controls resistance to P. syringae that express avrRpt2. Many F1 and F2 progeny exhibited intermediate resistance phenotypes. In addition to RPS2, at least one additional genetic interval associated with this defense response was identified and mapped using quantitative genetic methods. Further genetic and molecular genetic complementation experiments with cloned RPS2 alleles revealed that the Po-1 allele of RPS2 can function in a Col-0 genetic background, but not in a Po-1 background. The other resistance-determining genes of Po-1 can function, however, as they successfully conferred resistance in combination with the Col-0 allele of RPS2. Domain-swap experiments revealed that in RPS2, a polymorphism at six amino acids in the LRR region is responsible for this allele-specific ability to function with other host factors.

PLANT disease resistance is often controlled by gene-for-gene interaction between plant resistance (R) genes and pathogen avirulence (avr) genes (Crute and Pink 1996; Hammond-Kosack and Jones 1997). When R and avr alleles of matched specificity are present, the plant induces strong defense responses that restrict pathogen growth. This defense-inducing capacity is likely to require the action of many host factors in addition to the R gene product.

The interaction between R and avr gene products has often been modeled as a receptor-ligand interaction, and a small number of examples provide support for direct physical interaction (Scofield et al. 1996; Tang et al. 1996; Jia et al. 2000; Leister and Katafiri 2000). To date, new pathogen recognition specificities have most often been traced to variation within the leucine-rich repeat (LRR)-encoding domain of R genes, reinforcing the concept that the LRR is primarily a pathogen recognition domain (Parniske et al. 1997; Thomas et al. 1997; McDowell et al. 1998; Meyers et al. 1998; Ellis et al. 1999; Noel et al. 1999; Bittner-Eddy et al. 2000; Luck et al. 2000). A similar paradigm is well developed for LRR receptor proteins from mammals and other organisms (e.g., Braus et al. 1991; Kobe and Deisenhofer 1994; Marino et al. 2000). Individual plants carry hundreds of apparent R genes and substantial allelic diversity can exist among the LRR-encoding domains of R genes, giving rise to a wide array of pathogen recognition specificities (Ellis et al. 2000; Young 2000). Structural variation within other R gene domains and within pathogen avr alleles can also contribute to new pathogen recognition specificities (Herbers et al. 1992; Ellis et al. 2000; White et al. 2000).

A simple receptor-ligand model for the interaction of R and avr gene products does not preclude a requirement for additional host factors in defense signaling. These other host factors may act upstream, downstream, in parallel, or in concert with an interaction between R and avr gene products. In one example, the Rar1 gene is required for the function of some Mla R gene alleles in barley (Shirasu et al. 1999). Two tomato R gene products, the Pto kinase and the Prf nucleotide-binding site (NBS)-LRR protein, are both required for the resistance response against P. syringae pathogens that express avrPto (Martin et al. 1993; Salmeron et al. 1996), but physical interaction between the Pto and Prf proteins has not been reported. The presence of a high-affinity binding site for Avr9 peptide in both Cf-9* and Cf-9 tomato cell extracts suggests that other gene products are required for a defense-inducing interaction to take place between Cf-9 and Avr9 (Kooman-Gersmann et al. 1996).

In some cases, genes have been identified that contribute to defense signaling in multiple R/avr gene pathways. Prf of tomato is required for the function of both

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and the Pto homolog Fen, and thus is shared between two separate pathways that mediate responses to different ligands (Salmeron et al. 1994). EDS1, NDR1, PBS2, and PBS3 provide examples of Arabidopsis genes for which mutations disrupt multiple, but not all, gene-for-gene interactions (Innes 1998). Rcr loci are required for the function of tomato Cf-9 and Cf-2 R genes (Hammond-Kosack et al. 1994). The literature on classical resistance genetics and breeding contains many additional examples of “modifier” loci that alter the activity or quantitative strength of one or more resistance loci (Michelmore 1995; Crute and Pink 1996; Hammond-Kosack and Jones 1997). Hence the presence and strength of the defense response in a given gene-for-gene resistance pathway can be modulated by variation at avr genes, R genes, or accessory plant loci. However, the molecular basis of these defense-determining interactions remains poorly understood.

The disease resistance gene RPS2 of Arabidopsis thaliana blocks infection by Pseudomonas syringae pathogens that express the avirulence gene avrRpt2 (Kunkel et al. 1993; Yu et al. 1993). As part of this response, resistant plants develop the hypersensitive response, (HR), a programmed cell death process that arises within hours at and around the site of infection. The HR is associated with disease resistance in many gene-for-gene systems (Goodman and Novacky 1994; Greenberg 1997). Like many other R genes, RPS2 encodes a NBS-LRR protein (Bent et al. 1994; Mindinos et al. 1994; Young 2000). The present study was initially designed to identify additional host genes that function with RPS2 in defense activation. Ecotype Col-0 is RPS2/RPS2 and responds to P. syringae expressing avrRpt2 by inducing defense responses and limiting bacterial growth (Kunkel et al. 1993; Yu et al. 1993). The Arabidopsis ecotype Po-1 was previously identified as susceptible to P. syringae expressing avrRpt2 (Whalen et al. 1991), but the cause of susceptibility was not determined. Here we use genetic and molecular genetic analysis of Col-0 and Po-1 to show the involvement of one or more loci other than RPS2 in controlling the avrRpt2-specific resistance response. Allele-specific interactions were observed. We discovered that the LRR-encoding domain is the RPS2 determinant of allele-specific interactions between RPS2 and one or more of the other loci that participate in RPS2-mediated resistance.

**MATERIALS AND METHODS**

**Plant and bacterial strains: growth, inoculation, and transformation procedures:** P. syringae pv. tomato (Pst) DC3000 and P. syringae pv. glycinea Race 4 (Psg) carrying pVS61 (empty vector, no avr gene) or pV288 (pVS61 + avrRpt2) were constructed and used as described (Kunkel et al. 1993). Arabidopsis ecotype Col-0 was originally obtained from S. Somerville (Stanford University, Stanford, CA) and Po-1 was obtained from the former Arabidopsis Information Service seed bank (now available from ABRC, Columbus, OH; http://www.aims.cps.msu.edu/aims/). The Po-1 lines used in this study were derived from a line produced by two generations of single-seed descent. Arabidopsis plants were grown from seed in growth chambers under a 9-hr photoperiod at 22° and were moved after inoculation and scoring to a 24-hr photoperiod for flowering and seed production.

To assay for the HR, bacterial suspensions of ~2 × 10⁶ cfu/ml of Psg strains carrying pVS61 or pV288 were infiltrated into leaf mesophyll tissue by vacuum infiltration, with a disposable Pasteur pipette, or with a 1.0-ml syringe applied to the undersurface of healthy, fully expanded Arabidopsis leaves (Kunkel et al. 1993; Yu et al. 1993). Leaves were scored for HR symptoms at 24–48 hr after inoculation. To assay for disease, Pst bacterial suspensions of 5 × 10⁶ or 1 × 10⁷ cfu/ml in 10 mm MgCl₂ were infiltrated into plant leaves as described for the HR assay above (Whalen et al. 1991). The inoculated leaves were scored for disease symptoms (necrosis and yellowing) 4 days after inoculation. To determine levels of bacterial growth in the leaves of Arabidopsis, leaves of at least six plants per bacterial strain were vacuum infiltrated with bacterial suspensions of 2 × 10⁶ cfu/ml or 5 × 10⁶ cfu/ml. Bacterial growth was monitored by dilution plating of leaf samples at various time points between days 0 and 4 after inoculation as described previously (Whalen et al. 1991).

A modified vacuum infiltration procedure was used for transformation of Arabidopsis with constructs delivered by Agrobacterium tumefaciens strain GV3101(pMP90) (Bechtold et al. 1993; Clough and Bent 1998). Controls for experiments with transgenic plants included Po-1 and Col-0 ecotypes either grown on 0.5× MS/0.8% agarose medium without antibiotics and transplanted to soil or transformed with the parent binary cosmid pCLD04541 (Bancroft et al. 1997), selected on antibiotic media, and transplanted to soil.

**Genetic linkage analysis:** Genetic mapping with Po-1 × Col-0 F₁ individuals and F₃ families was performed using the indicated cleaved amplified polymorphic sequence (CAPS), simple sequence length polymorphism (SSLP) markers (Research Genetics, Huntsville, AL), and restriction fragment length polymorphism (RFLP) markers (ABRC) that map throughout the Arabidopsis genome (Nam et al. 1989; Konieczny and Ausubel 1993; Bell and Ecker 1994; Rhee et al. 1998; http://www.arabidopsis.org/). For plant genomic DNA, one to two inner rosette leaves from F₂ plants, or ~1 g fresh weight of leaves from ≥30 F₃ plants, were collected after testing plants for the HR phenotype, immediately frozen in liquid N₂, and stored at −70°. Genomic DNA was isolated using a CTAB-based protocol (Rogers and Bendich 1988). PCR for genetic mapping was essentially as described (Konieczny and Ausubel 1993; Bell and Ecker 1994). For the RPS2 CAPS, a portion of RPS2 was amplified using primers 5′-CAG AGC TTT GAG ACA G-3′ and 5′-GTA CTC CAA GTC ATG-3′, and an aliquot of the PCR product was digested with restriction enzyme EcoRI and resolved by agarose gel electrophoresis. The 16 individuals mentioned as the “biased mapping set” were screened by selecting a total of 785 Po-1 × Col-0 F₂ individuals by hand inoculation with Psg avrRpt2 to test for the HR and by genotyping at RPS2 using the EcoRI-based CAPS marker. Unless otherwise noted, molecular biological methods used in these and other experiments were essentially as described (Ausubel et al. 1997).

In initial mapping studies, significant associations between marker and defense phenotype were assessed using 57 susceptible F₂ individuals using the chi-square statistic to test for deviation from a 3:1 or 12:3 ratio (P ≤ 0.05). Statistically significant associations were observed between the resistance phenotype and the three markers nga8, RPS2, and DHS1A.

More detailed genetic mapping was performed using “set I” (131 F₂ families derived by self-fertilization from randomly
chosen Po-1 × Col-0 F1 individuals from 5 different F1 plants) separately or with “set II” (16 F1 lines from the biased mapping set described above and 53 F1 families derived from other Po-1 × Col-0 F1 individuals homozygous at RPS2). Phenotypes of the F2 families were determined using at least two separate pots, each containing ≥98 and typically 14 or more plants from each F2 family. Plants were inoculated with Psg avrRpt2+ by vacuum infiltration and before viewing of labels the set of F2 plants in a pot were assigned a single group score for severity of the HR on a scale of 0–4. Each infiltration set included one pot each of Col-0 and Po-1 as controls. The following categories were used: (1) no HR, all leaves on all plants show no HR or at most HR1; (2) rare and/or weak HR, most leaves do not show extensive tissue collapse, a few leaves may show HR3, with most leaves showing HR1–2; (3) intermediate HR, most leaves on all plants show an intermediate HR2 or HR3, with some leaves showing HR4; (4) full HR, all leaves on all plants show extensive tissue collapse (HR4–5); (5) segregating) majority of plants show HR4–5 but some plants show no HR or intermediate HR (HR0–3). After being placed into these categories without reference to labels, variation of HR within an F2 family was evaluated by comparing the response of the plants between duplicate pots of the same F2 family. As a check, independent scoring of selected experiments by other laboratory personnel produced consistent categorization of F2 families.

F2 mapping data were analyzed using QGene v3.06 (Nelson 1997), with map distances for molecular marker maps obtained from the Lister and Dean RI map (Rhee et al. 1998; http://www.arabidopsis.org). Single interval mapping protocols were used and significance of association between marker structs that were independently tested in plants.

DNA sequencing: The DNA sequence of Po-1 RPS2 was determined for both strands usingideo sequencing methods and RPS2 internal primers. One PCR product amplified from genomic Po-1 DNA and cloned into pBluescript II SK (+) was used for initial sequencing. This PCR product was generated using the primers aa#1 (5′-GGAGATCCGATTATCTGGTGGG-3′) and 46S (5′-ACAGATGTACTTACATAA-3′). Any deviations from the known Col-0 RPS2 sequence were then checked using independent Po-1 RPS2 PCR products. Note that no introns are present in Col-0 or Po-1 RPS2. The promoter region of Col-0 RPS2 was cloned from a genomic subclone (Bent et al. 1994) as a 1.3-kb SacI-BamHI fragment into pBluescript II SK (+); the promoter region of Po-1 RPS2 was cloned from a PCR product generated using RPS2-P1k-Cla (5′-CGGAATTCAGTTCTTTATCTCATCTCTCTTTTTTA-3′) and RPS2-sal6 (5′-GATTTCTCGCTC-3′). Any deviations from the known Col-0 RPS2 sequence were then checked using independent Po-1 RPS2 PCR products. Note that no introns are present in Col-0 or Po-1 RPS2. The promoter region of Col-0 RPS2 was cloned from a genomic subclone (Bent et al. 1994) as a 1.3-kb SacI-BamHI fragment into pBluescript II SK (+); the promoter region of Po-1 RPS2 was cloned from a PCR product generated using RPS2-P1k-Cla (5′-CGGAATTCAGTTCTTTATCTCATCTCTCTTTTTTA-3′) and RPS2-sal6 (5′-GATTTCTCGCTC-3′). Any deviations from the known Col-0 RPS2 sequence were then checked using independent Po-1 RPS2 PCR products.

RESULTS

Response of Po-1 to P. syringae expressing avrRpt2: To investigate the response of Po-1 to infection by P. syringae expressing avrRpt2, leaves of Po-1 and Col-0 were inoculated by syringe or by vacuum infiltration with the virulent Pst strain DC3000 or with Pst DC3000 expressing avrRpt2 (DC3000avrRpt2+). Wild-type Col-0, which is resistant to avrRpt2, developed few or no disease symptoms when inoculated with Pst DC3000avrRpt2+ at a titer of 106 colony-forming units (cfu)/ml (Table 1). In confirmation of previous work (Whalen et al. 1991), Po-1 plants developed necrotic lesions and pronounced chlorosis 4 days after inoculation with DC3000avrRpt2+, which are similar to the symptoms observed on susceptible Col-0 rps2/rps2 mutants or on wild-type Col-0 inoculated with DC3000 (Table 1). The lack of a resistance response in Po-1 was quantified by measuring the extent of pathogen growth within the plant. In Po-1 inoculated with either DC3000 or DC3000avrRpt2+, bacteria grew to high levels (Figure 1). These levels were similar to those attained by
TABLE 1
Response of Arabidopsis ecotypes Po-1, Col-0, and Col-0 rps2/rps2 to P. syringae that express avrRpt2

<table>
<thead>
<tr>
<th>Plant line (genotype)</th>
<th>HR score at 24 hr: Psg R4avrRpt2</th>
<th>Disease score at 96 hr: Pst DC3000 Pst DC3000avrRpt2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (RPS2/RPS2)</td>
<td>HR+ 4.0–5.0</td>
<td>Disease 3.5–5.0</td>
</tr>
<tr>
<td>D203 (Col-0 rps2/rps2)</td>
<td>No HR 0.0–1.0</td>
<td>Disease 4.0–5.0</td>
</tr>
<tr>
<td>Po-1 (?/?</td>
<td>No HR 0.0–1.0</td>
<td>Disease 3.5–5.0</td>
</tr>
</tbody>
</table>

Each entry reports results from at least three experiments with five or more plants per ecotype per experiment; numbers are the range of mean scores for independent experiments. For the HR assay, bacteria were inoculated at \(2 \times 10^8\) cfu/ml. Hypersensitive response was scored on a scale of 0–5. HR score \(\leq 1.5\): little or no HR, no visible tissue collapse. \(>3.5\): intermediate HR. For disease assay, bacteria were inoculated at \(2 \times 10^6\) cfu/ml. Disease was evaluated by symptoms of chlorosis and small necrotic lesions, and scored on a scale of 0–5. Disease score \(\leq 1.5\): little or no disease. 1.5–2.5: mild disease. 3.0–4.0: moderate disease. \(\geq 4.0\): severe disease.

DC3000avrRpt2\(^{+}\) in susceptible rps2 mutants of Col-0 or by DC3000 (no avr) in wild-type Col-0. In contrast, growth of DC3000avrRpt2\(^{+}\) in wild-type Col-0 plants was restricted, reaching maximum levels of \(10^4–10^5\) cfu/cm\(^2\) (Figure 1).

The hypersensitive response (HR) is a programmed cell death response that develops within hours at and around the site of infection. The ability of Po-1 to develop an HR in response to P. syringae pv. glycinea (Psg) expressing avrRpt2 was tested by syringe or vacuum infiltration with a high titer of bacteria \((10^8\) cfu/ml; Klement et al. 1964). While Col-0 plants exhibited a strong, visible HR within 24 hr of inoculation, Po-1 plants did not manifest an HR at the macroscopic level in response to Psg avrRpt2\(^{+}\) (Table 1). Po-1 plants do have the capacity to induce gene-for-gene defenses and the HR in response to P. syringae pathogens, however, as Po-1 activates these responses when inoculated with P. syringae that express avrRps4 (Hinsch and Staskawicz 1996; data not shown). Although the HR is not always required for an effective resistance response (Yu et al. 1998; Bendahmane et al. 1999), it is closely associated with the disease resistance response mediated by RPS2 and most other R genes (Kunkel et al. 1993; Yu et al. 1993; Goodman and Novacky 1994; Greenberg 1997). In this study the level of the HR was frequently used as an indicator of the avrRpt2-RPS2 resistance response of the plant.

To summarize, Col-0 plants inoculated with P. syringae expressing avrRpt2 developed an HR, restricted pathogen growth, and did not develop disease. In response to the same bacteria Po-1 plants did not manifest an HR, limited pathogen growth poorly, and developed disease. The simplest explanation for Po-1 susceptibility to P. syringae that express avrRpt2 would be that Po-1 carries a nonfunctional allele of RPS2.

**Multigenic control of RPS2-mediated defense:** The genetic basis of susceptibility in Po-1 was investigated by crossing ecotypes Po-1 and Col-0. Po-1 × Col-0 F\(_1\)
TABLE 2
HR in response to Pst R4 \textit{avrRpt2} in F1 from crosses involving Po-1

<table>
<thead>
<tr>
<th>Cross (including reciprocal)</th>
<th>No. of F1 tested</th>
<th>Mean HR score (and phenotype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Po-1 × Col-0</td>
<td>n = 18</td>
<td>4.68 ± 0.16 (HR+)</td>
</tr>
<tr>
<td>Po-1 × No-0</td>
<td>n = 26</td>
<td>4.74 ± 0.15 (HR+)</td>
</tr>
<tr>
<td>Po-1 × D203</td>
<td>n = 27</td>
<td>0.15 ± 0.14 (HR+)</td>
</tr>
<tr>
<td>Po-1 × 101C</td>
<td>n = 29</td>
<td>0.60 ± 0.16 (HR+)</td>
</tr>
</tbody>
</table>

For the HR assay, bacteria were inoculated at $2 \times 10^8$ cfu/ml. Hypersensitive response was scored on a scale of 0–5 ± SE. HR score ≤1.5: little or no HR, no visible tissue collapse. ≥3.5: HR+, extensive cell death, obvious collapse of inoculated tissue. >1.5 and <3.5: intermediate HR. For each entry, data were pooled for multiple crosses including reciprocal crosses.

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FIGURE 2.—Phenotypic segregation in F2 and F3 progeny of Po-1 crossed to various genotypes, as indicated. A and B represent different F2 populations, with A subjected to the HR assay and B subjected to the disease assay (see MATERIALS AND METHODS). C–E also report results of HR assays; C reports disease-susceptible or HR2 class of F2 individuals were data for F3 families rather than for F2 individuals.

were not consistent with the segregation of a single dominant \textit{R} gene or with standard ratios for digenic inheritance, such as 9:7 or 9:3:4, the data also did not resemble the bell-shaped curves that are often observed in F2 populations segregating for a quantitative trait controlled by a large number of genes displaying small additive effects (\textit{Falconer} and \textit{Mackay} 1996). Instead, the bimodal distribution of Po-1 × Col-0 and Po-1 × No-0 F2 and F3 phenotypes indicated that resistance segregates as a multigenic trait controlled by a small number of major-effect genes or by a single dominant gene and a small number of "modifier" genes.

\textbf{Genetic evidence for Po-1 \textit{RPS2} functionality:} To determine whether the \textit{RPS2} allele of Po-1 is compromised for response to \textit{avrRpt2}, the \textit{RPS2} genotype was determined for F2 lines that were also scored for resistance phenotype (Table 3). A single-base pair \textit{EcoRI} CAPS within \textit{RPS2} was identified that differentiates the \textit{RPS2} alleles of Col-0 and Po-1. F2 individuals were identified that are homozygous for the Po-1 \textit{RPS2} allele, yet they...
Numbers shown are the number of randomly chosen F2 individuals in each genotypic class. HR assay and scoring are as described in MATERIALS AND METHODS. Underlined numbers represent particularly informative classes (see text).

showed a partially or fully disease-resistant phenotype (Table 3). These F2 individuals suggested that, despite the lack of avrRpt2-specific resistance in wild-type Po-1, the Po-1 RPS2 allele can function in a partial Col-0 background. Another class of F2 individuals was homozygous for the Col-0 RPS2 allele but showed little or no disease resistance (Table 3). These individuals indicated that other Po-1 loci can cause functional RPS2 alleles to be ineffective for resistance signaling in response to avrRpt2. Results consistent with these F2 data were obtained in repeat assays with F2 families derived from the key F2 lines and in 16 additional F2 individuals identified among 785 Po-1 × Col-0 F2 (see MATERIALS AND METHODS). To reiterate, these classes of F2 RPS2 homozygotes indicated that the Po-1 allele of RPS2 can be functional and/or that the progeny of Po-1 × Col-0 crosses segregate for genes other than RPS2 that control disease resistance.

In Po-1 × Col-0 F2 populations, the defense phenotype did not segregate independently of the RPS2 genotype (Table 3). F2 plants homozygous for the Col-0 RPS2 allele were most frequently resistant and F2 plants homozygous for the Po-1 RPS2 allele were most frequently susceptible. Because resistance/susceptibility did not segregate entirely independently of the RPS2 genotype, we hypothesized that one or more of the other resistance-modifying genes is linked to RPS2. A separate but not mutually exclusive hypothesis was that RPS2 is one of the genes contributing to the avrRpt2-specific resistance response, with allele-specific interactions causing the presence or absence of resistance.

Mapping of RPS2-pathway loci: An approximate map position for one or more other RPS2-pathway loci that alter the defense response against P. syringae that express avrRpt2 was determined using a population of 131 random F2-derived F1 families from crosses between Po-1 and Col-0. A second population of 69 F2 families contained a small biased population of 16 lines in which the resistance phenotype was the opposite of that predicted by the RPS2 genotype (e.g., underlined classes in Table 3), as well as 55 other F2 families not from set 1 and chosen due to homozygosity at RPS2. Plants were inoculated with Psg avrRpt2 by vacuum infiltration and scored for the HR. Previously mapped CAPS or RFLP markers were used to determine genotype across the Arabidopsis genome with genetic intervals of 50 cM or less.

Analysis of the initial marker data set revealed linkage of the avrRpt2-specific response to at least two regions on chromosome 4, near markers nga8, RPS2, and DHS1A, and detected no linkage to chromosomes 1–3 or 5 (data not shown). The additional F2 lines and additional chromosome 4 markers were subsequently used for higher resolution mapping. Quantitative trait statistical analysis of the marker data, using single-interval mapping methods, localized genetic determination of the avrRpt2-specific response to two discrete genetic intervals (Figure 3). The strongest effect was at the RPS2 locus. A second locus that contributed to the avrRpt2-specific response was linked to marker DHS1, roughly 33 cm away from RPS2. The possibility that additional loci linked to RPS2 on chromosome 4 also contribute to this phenotype cannot be excluded. No linkage association was detected between the defense trait and any markers on chromosomes 1–3 or 5 (Figure 3).

Allele-specific functionality of RPS2: The discovery of Po-1 × Col-0 F2 individuals that are homozygous for the Po-1 allele of the RPS2 allele but that show a resistant phenotype suggested that the Po-1 RPS2 allele can be functional when it is in a partially Col-0 background. Functionality of Po-1 RPS2 was investigated further by testing for the resistance response of plants carrying the Po-1 RPS2 allele in a Col-0 rps2/ rps2 background. In a genetic approach, Po-1 was reciprocally crossed with Col-0 mutants rps2-201/rps2-201 (D203) and rps2-101C/rps2-101C (101C). The rps2-201 allele carries a point mutation that causes a single-amino-acid change in the LRR and creates a nonfunctional RPS2 protein, while the rps2-101C allele contains a frame-shift mutation that causes a premature stop codon at the front of RPS2 (BENT et al. 1994; Mindrinos et al. 1994). In the progeny of D203 or 101C crosses to Po-1, all F1 were HR+ (Table 2). However, ~70% of the F2 showed an intermediate or strong HR (Figure 2E; data not shown). These results again suggested (see also Table 3) that the Po-1 allele of RPS2 is functional when moved into a partially Col-0 genetic background but cannot signal for resistance in conjunction with Po-1 alleles of these resistance-modifying loci.

An alternative hypothesis to explain these results was that Po-1 genes other than RPS2 are capable of mediating the HR in conjunction with Col-0 genes other than RPS2. To test this hypothesis, we investigated whether any HR+ individuals were homozygous for the nonfunctional Col-0 rps2-201 or rps2-101C mutant alleles of RPS2. The RPS2 genotype was determined for all F2 progeny
that showed an intermediate or strong HR, and all 56 HR\textsuperscript{+} F\textsubscript{2} individuals carried at least one copy of the Po-1 RPS2 allele (data not shown). This suggested that Po-1 RPS2 is the cause of avrRpt2-specific resistance signaling in these lines. However, because of possible contributions from loci tightly linked to RPS2, this result still did not conclusively rule out the possibility that resistance is mediated by interaction among genes other than RPS2.

Functionality of Po-1 RPS2 was investigated more precisely by molecular complementation. The Po-1 RPS2 allele under \~1.0 kb of native Po-1 RPS2 promoter was cloned into a binary cosmid and transferred by Agrobacterium-mediated transformation into the Col-0 rps2/ rps2 mutants D203 and 101C. Transformants were found to produce a resistance response upon challenge with Psg avrRpt2, indicating that the Po-1 RPS2 allele can be functional in a Col-0 genetic background (Figure 4a). It was noted, however, that the HR in these lines was intermediate in intensity.

In a reciprocal experiment, the Col-0 RPS2 allele under 1.6 or 1.0 kb of native promoter was transformed into Po-1 plants. The Col-0 RPS2 allele complemented Po-1 to resistance in response to Psg avrRpt2\textsuperscript{+} (Figure 4A). This complementation result was significant, as it indicated that the absence of avrRpt2-specific resistance in Po-1 is due not only to defects at other loci, but also to the Po-1 allele of RPS2.

To summarize the above genetic and molecular genetic complementation experiments, allele-specific interactions were observed between RPS2 and one or more other loci. Col-0 RPS2 could function with the Po-1 allele of one or more genes other than RPS2 that control avrRpt2-specific disease resistance, while RPS2 from Po-1 did not function with the Po-1 alleles of these other genes. Po-1 RPS2 did function with the Col-0 alleles of these other genes, as did Col-0 RPS2. The Po-1 alleles of RPS2 and this other gene or genes are each capable of disease resistance function, but they cannot function with each other.

Sequence of Po-1 RPS2 allele: To investigate possible structural differences between the Po-1 and Col-0 RPS2 alleles that might account for their differences in resistance signaling, the Po-1 allele of RPS2 was cloned and sequenced (GenBank accession no. AF368301). The derived amino acid sequence revealed a substantial number of differences—11 amino acid changes—between the Po-1 and Col-0 RPS2 alleles (Figure 5). Many of the nonconservative amino acid changes are located in the leucine-rich repeat (LRR) region, but residue changes are scattered over much of the RPS2 ORF. The derived amino acid sequence did not reveal obvious structural features that might suggest that the Po-1 allele of RPS2 is nonfunctional.

No transcriptional differences between Col-0 and Po-1 RPS2 alleles: Previous Northern analysis of RPS2 mRNA from noninoculated Po-1 and Col-0 leaf tissue
did not show a discernible difference in expression between the Po-1 and Col-0 RPS2 transcripts (Bent et al. 1994), suggesting that differences in the level of transcription do not account for the difference in resistance signaling activity between Po-1 and Col-0 RPS2 alleles.

To further investigate whether transcriptional differences between Po-1 and Col-0 RPS2 transcripts are responsible for the difference in defense signaling activity of the two alleles, the RPS2 promoter sequences were investigated. Approximately 1.0 kb of genomic DNA immediately upstream of the Col-0 and Po-1 RPS2 open reading frames was cloned, sequenced, and compared. Across this 986-bp sequence, the Po-1 RPS2 promoter differed from the Col-0 RPS2 promoter at only 7 bp positions, none obviously disrupting a promoter motif (see GenBank accession nos. AL049483 and AF368301).

Figure 5.—Derived amino acid sequence encoded by RPS2 of Po-1. Differences with Col-0 RPS2 are in boldface type and the Col-0 amino acid is shown directly above. Lines indicate the approximate extent of putative functional domains; broken lines for the leucine-rich repeat reflect the imperfect nature of the LRR in the RPS2 gene product. ***, HindIII site that formed junction for LRR-swap alleles (see Figure 4).

Figure 4.—Molecular complementation experiments using cloned RPS2 constructs. Values shown are mean ± SE for severity of HR in multiple T1 transformants tested for their response to Psg R4 avrRpt2. Plants were transformed with the following: (A) an intact RPS2 gene driven by 1.0 kb of native RPS2 promoter from the genotype indicated; (B) an intact RPS2 open reading frame driven by 1.0 kb of native promoter or by heterologous RPS2 promoter from a different genotype, as indicated; (C) RPS2 LRR-swap constructs fusing promoter and amino-terminus-encoding region from one RPS2 allele with the LRR-encoding region from a heterologous RPS2 allele, as indicated. ϕ, plants transformed with vector (no RPS2 insert), or, in some cases, nontransformed plants carried through growth and transplanting in parallel with transformants but on nonselective media.
promoter in the Col-0 or Po-1 backgrounds (Figure 4B). These results provided functional evidence that Po-1 and Col-0 RPS2 promoters do not differ in any appreciable manner that might account for differences in the phenotypic expression of RPS2-mediated defense responses.

Differences responsible for allele-specific interaction are in the LRR domain: R gene products contain identifiable motifs such as a coiled-coil domain, NBS, and LRR (Hammond-Kosack and Jones 1997; Young 2000). We pursued further domain-swap experiments to determine if functional differences between the Po-1 and Col-0 alleles of RPS2 could be assigned to amino acid differences in a given domain.

The Po-1 and Col-0 alleles of RPS2 under the control of 1.0 kb of native promoter in a binary vector were used as the parent constructs. From the parent constructs, the 3’ 1.35-kb fragment of Po-1 RPS2 encoding the LRR was cloned out and replaced with the 3’ 1.35-kb fragment of Col-0 RPS2 and vice versa. The chimeric LRR-swap constructs were transformed into Po-1 and into the Col-0 rps2/rps2 mutants D203 and 101C by Agrobacterium-mediated transformation, and transformants were tested for their HR in response to Psg avrRpt2. We found that the Po-1 amino terminus + Col-0 LRR constructs could mediate an intermediate level of HR in Po-1 and in Col-0 rps2/rps2 genetic backgrounds, indicating that the amino terminus of Po-1 RPS2 can function even in a Po-1 genetic background (Figure 4C). The Col-0 amino terminus + Po-1 LRR constructs, on the other hand, mimicked the results obtained with intact Po-1 RPS2: an intermediate HR was observed in Col-0 rps2/rps2 genetic backgrounds, and no HR was conferred in a Po-1 genetic background (Figure 4C). The Col-0 RPS2 LRR domain corrected the nonfunctional Po-1 RPS2 LRR domain for resistance in a Po-1 genetic background. This indicated that the LRR domain is the key structural determinant for allele-specific interactions between RPS2 and other host loci that modify the avrRpt2/RPS2 pathway in this Col-0/Po-1 system.

**DISCUSSION**

This study explored the interaction of RPS2 with other hypothesized host factors required for the perception of *P. syringae* pathogens that express avrRpt2 and/or for the subsequent induction of plant defense responses. Progeny of crosses between a resistant and a susceptible ecotype of Arabidopsis revealed segregation of more than one gene controlling this defense response. Polymorphism between the Po-1 and Col-0 alleles of RPS2 was a major factor determining the strength of the avrRpt2-specific resistance response, but it was not the only factor. At least one additional genetic interval that contributes to this phenotype was identified and mapped. We discovered that Po-1 RPS2 can function in a Col-0 genetic background, but not in Po-1. In RPS2, the LRR domain was responsible for ineffective interaction between Po-1 RPS2 and one or more of the other Po-1 loci.

**Roles of the LRR:** LRR domains are found in a wide array of proteins from all taxa and are present in almost all structural classes of plant R genes that mediate gene-for-gene disease resistance (Kobe and Deisenhofer 1994; Hammond-Kosack and Jones 1997; Marino et al. 2000). LRRs are involved in the perception of protein or peptide ligands in a number of systems, including interactions between the Drosophila Toll receptor and the dorsal/ventral patterning factor Spatzle; human follicle stimulating hormone and its receptor; and among plant development proteins such as CLAVATA1, 2, and 3 (Kobe and Deisenhofer 1994; Fletcher et al. 1999; Marino et al. 2000). However, LRRs have also been shown to mediate intracellular interactions among proteins not thought of as “receptors” and “ligands,” such as yeast adenylate cyclase and Ras (e.g., Suzuki et al. 1990).

In plant R gene products, studies suggest that the LRR domains are major determinants of recognitional specificity for Avr factors (Ellis et al. 2000). Evolution of new pathogen specificity has been traced to shifts in solvent-exposed LRR residues that are caused by single-base changes, insertion or deletion events, and by equal- or unequal-exchange meiotic recombination events within *R* genes or between closely linked homologous *R* genes in a cluster (Ellis et al. 2000).

Roles other than pathogen recognition have also been hypothesized for the LRR of *R* gene products, but these have been less clearly demonstrated. In this study we obtained evidence that the LRR region can influence effective interaction with host factors. Consistent with our results, a study with the Arabidopsis *R* gene RPS5 also suggested a role for the LRR domain in interaction with other host factors (Warren et al. 1998). A nonfunctional RPS5 allele containing a mutation in the third repeat of the LRR blocked the resistance conferred by other *R* genes, and overexpression of wild-type RPS5 did not suppress the dominant-negative phenotype of the mutant allele (Warren et al. 1998). This RPS5 mutation of the third LRR might have caused increased binding to a pathway component(s) shared by multiple *R* genes and thereby interfered with essential downstream signaling. In our study, the difference in interaction between Col-0 and Po-1 RPS2 and other host loci was attributed to six amino acid differences between the RPS2 LRR domains. In the future, it will be interesting to see whether amino acid polymorphisms within the LRR of RPS2 alleles from other ecotypes correlate with the level of the resistance response.

The RPS2 and RPS5 examples fit into a generalized model proposed by Grant and Mansfield (1999) to account for the involvement of additional loci in *R-Avr* interactions. In their model, the LRR protein only indirectly matches the *Avr* protein and is involved in inter-
preventing signals generated from other cellular proteins, designated signaling linker proteins (SLIKs), which directly interface with the Avr peptide. The presence of the elicitor or Avr factor, or its activity, may alter the normal configuration of the SLIK or SLIK complex, leading to functional interaction with the R gene product and subsequent resistance pathway activation (Grant and Mansfield 1999). The interactions that we observed involving avrRpt2, RPS2, and other host factors may, upon further investigation, form one example of this type of SLIK interaction.

**RPS2-interacting loci**: As an initial step toward isolation of the RPS2-interacting host factors predicted by our genetic studies, quantitative trait methods were used to map genetic intervals associated with the avrRpt2-specific response. The bimodal distribution of resistance phenotypes among Po-1 × Col-0 and Po-1 × No-0 F2 (Figure 2) classically would indicate that the phenotype, in this case resistance in response to avrRpt2, is controlled by a small number of major-effect genes or a single dominant gene and a small number of “modifier” genes. The observed bias toward defense phenotypes that correlated with the RPS2 genotype (HR+ if homozygous for Col-0 RPS2, HR− if homozygous for Po-1 RPS2; see Table 3) had suggested that RPS2 would have a significant phenotypic effect and/or that other relevant loci would be linked to RPS2. Mapping supported both hypotheses. The defense phenotype associated most strongly with the RPS2 locus, which was also shown by other means to have a major effect on resistance phenotypes (Figure 3). The other genetic interval associated with the response to *P. syringae* that express avrRpt2 also mapped to chromosome 4, ~33 cM away from RPS2. As mentioned previously, the possibility that additional loci linked to RPS2 on chromosome 4 also contribute to this phenotype could not be excluded.

Reports of multigenic control of resistance are gaining relevance in research on the molecular basis of defense signal transduction as resources improve for the mapping and cloning genes known only by phenotypic type. A number of other Arabidopsis genes have been identified for which mutant alleles disrupt defense pathways (Glazebrook 1999). None of the well-studied genes (such as NDR1, EDS1, PAD4, DND1, LBD1, and PBS2) map to the intervals on chromosome 4 identified in this study. Further experimental effort will be required to isolate and characterize the RPS2-interacting host factor(s) described in this study.

Direct protein associations among host factors known to be required for the *R*-*avr* signaling complex have yet to be demonstrated. In the closest example to date, Pto kinase has been shown to directly phosphorylate Pti1 (Zhou et al. 1995). In a more immediate example, Lester and Katagiri (2000) used AvrRpt2 to coprecipitate RPS2 and another unidentified protein in antibody pull-down experiments. Interestingly, RPS2 could also be precipitated by AvrB despite the fact that RPS2 does not confer resistance to *P. syringae* that express avrB (Leister and Katagiri 2000). This result is consistent with genetic evidence for interference between RPS2 and RPM1 resistance signaling pathways when pathogens that express avrB or avrRpm1 and avrRpt2 are co-inoculated (Reuber and Ausubel 1996; Ritter and Dangl 1996).

Although the interacting loci found in this study are characterized as defense pathway loci, it is also possible that these loci are active in disease susceptibility. avrRpt2 has been shown to promote virulence in the absence of RPS2 (Chen et al. 2000), and one or more of the loci identified in this study may encode a protein that is a target for the virulence activity of AvrRpt2.

**Allele-specific interactions**: The strong resistance response of ecotype Col-0 to *P. syringae* that express avrRpt2 is known to be dependent on RPS2 (Kunkel et al. 1993; Yu et al. 1993). The lack of an effective response in Po-1 initially suggested that Po-1 does not carry a functional RPS2 allele. We discovered that Po-1 carries an allele of RPS2 that confers avrRpt2-specific resistance in other genetic backgrounds, implying that defects in other Po-1 loci cause loss of RPS2 function. Intriguingly, the Col-0 RPS2 allele under native RPS2 promoter complemented Po-1 for resistance when introduced by transformation, suggesting that Po-1 LRR is also partly responsible for the nonfunctional resistance in Po-1. As noted above, we found that the LRR is the domain responsible for the RPS2 component of these allele-specific interactions.

Allele-specific interactions were not confined to the Po-1 allele of RPS2. The discovery of Po-1 × Col-0 F2 individuals and F3 families that were homozygous for Col-0 RPS2 but disease susceptible indicated that, in certain mixed Po-1/Col-0 genetic backgrounds, allele-specific interactions among resistance-modulating loci could also prevent resistance signaling through the otherwise functional Col-0 RPS2. The fully resistant phenotype of the Po-1 × Col-0 F1 indicated that the nonproductive interaction between alleles that prevent Col-0 RPS2 function is recessive.

In contrast to the above, nonproductive interactions were dominant when we monitored interaction between Po-1 RPS2 and the RPS2-interacting loci. The F1 of Po-1 × Col-0 rps2/rps2 mutants were HR− (Table 2). Po-1 RPS2 could function in concert with the Col-0 alleles at these other loci (Figure 4A), but could not function in the heterozygous background of these F1.

As a separate matter, we were intrigued that complementation experiments involving all or part of Po-1 RPS2 often produced a weak or intermediate HR (Figure 3). Our interpretation of this result is that Po-1 RPS2 (including the Po-1 amino terminus/Col-0 LRR fusion), even when functional, cannot interact with other host factors as effectively as Col-0 RPS2. It may also be the case that Po-1 RPS2 does not recognize the avrRpt2 ligand as effectively. Although some quantitative
reduction in responsiveness to the \textit{avrRpt2} ligand cannot be excluded, the constructs containing domains from Po-1 \textit{RPS2} could clearly mediate responses to \textit{P. syringae} that express \textit{avrRpt2}. In contrast, the host genotype at loci other than \textit{RPS2} had a pronounced effect, correlating with the presence or near-complete absence of a response to pathogen (Figure 4).

A separate example of allele-specific interactions that affect expression of resistance was recently provided by the demonstration of monogenic and novel digenic resistance mediated by three \textit{RXC} loci in the Arabidopsis ecotypes Col-0 and Landsberg erecta (Ler) in response to the bacterial pathogen \textit{Xanthomonas campestris} (Buell and Somerville 1997). In the \textit{RXC} defense system, monogenic resistance is determined by the presence of the Col-0 allele of \textit{RXC2} while in its absence, digenic resistance is specified by the presence of the Col-0 allele of \textit{RXC4} in conjunction with the Ler allele of \textit{RXC3}. Numerous combinations of the six \textit{RXC} alleles were shown to confer intermediate levels of resistance (Buell and Somerville 1997). The lack of resistance in Po-1 carrying Po-1 \textit{RPS2}, and in some mixed Col-0/Po-1 backgrounds carrying Col-0 \textit{RPS2}, may or may not have a similar molecular basis as the allele-specific interactions observed for \textit{RXC} loci.

In studies on the lesion mimic Arabidopsis mutant \textit{cep}, mapping crosses between genetically heterogeneous ecotypes showed that expression of the mutant phenotype was conditioned not only by the \textit{cep} locus but also by two other loci that were designated \textit{CPR20} and \textit{CPR21} (Silva et al. 1999). \textit{CPR20} mapped to the lower arm of chromosome 4 and was required for the \textit{cep} phenotype, while \textit{CPR21} of chromosome 1 was often but not always required for the \textit{cep} phenotype (Silva et al. 1999). The genetic interval encompassing \textit{CPR20} does not overlap with the genetic intervals on chromosome 4 that were found to contribute to the \textit{avrRpt2} resistance phenotype.

Sequence differences among \textit{R} gene alleles have been shown to cause quantitative variation in the defense response in many systems (reviewed in Ellis et al. 2000). The general finding of quantitative variation in defense responses has been observed in many additional disease resistance systems (Michelmore 1995; Crute and Pink 1996). Our study highlights the fact that this variation can be due as much to altered interaction among host factors as to altered interaction between \textit{R} gene product and pathogen-derived elicitors. Responsiveness to \textit{P. syringae} that express \textit{avrRpt2} could be observed with all natural and synthetic alleles of \textit{RPS2} that were studied. Allele-specific interaction between other host factors and the LRR domain of \textit{RPS2} played the primary role in determining whether or not gene-for-gene defense responses were triggered.

In the future, it should be particularly informative to isolate and characterize the \textit{RPS2/avrRpt2} pathway gene(s) implicated by this study, and to determine the precise structural determinants that control effective interaction between the \textit{RPS2} protein and its interacting host factors.

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