The Plant Cell, Vol. 10, 1439–1452, September 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

A Mutation within the Leucine-Rich Repeat Domain of the Arabidopsis Disease Resistance Gene *RPS5* Partially Suppresses Multiple Bacterial and Downy Mildew Resistance Genes

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Recognition of pathogens by plants is mediated by several distinct families of functionally variable but structurally related disease resistance (*R*) genes. The largest family is defined by the presence of a putative nucleotide binding domain and 12 to 21 leucine-rich repeats (LRRs). The function of these LRRs has not been defined, but they are speculated to bind pathogen-derived ligands. We have isolated a mutation in the Arabidopsis *RPS5* gene that indicates that the LRR region may interact with other plant proteins. The *rps5-1* mutation causes a glutamate-to-lysine substitution in the third LRR and partially compromises the function of several *R* genes that confer bacterial and downy mildew resistance. The third LRR is relatively well conserved, and we speculate that it may interact with a signal transduction component shared by multiple *R* gene pathways.

INTRODUCTION

The molecular recognition of pathogens by plants is often characterized by a gene-for-gene relationship that requires a specific plant resistance (*R*) gene and a corresponding pathogen avirulence (*avr*) gene (Flor, 1971). Genetic evidence from a wide diversity of plant pathosystems suggests that when an appropriate *R*–*avr* gene pair is present, the result is host resistance, whereas absence or inactivation of either member of the gene pair results in susceptibility of the host to the pathogen. A common explanation for the molecular basis of this gene-for-gene relationship is an elicitor–receptor model (Gabriel and Rolfe, 1990). According to this model, *avr* genes directly or indirectly produce an elicitor that is recognized by the corresponding *R* gene–encoded receptor. This molecular interaction then triggers downstream signaling events that result in the activation of plant defenses and the limitation of pathogen growth.

R genes have been cloned from several plant species (reviewed in Bent, 1996; Baker et al., 1997; Hammond-Kosack and Jones, 1997). These include *R* genes that mediate resistance to bacterial, fungal, oomycete, viral, and nematode pathogens. Many of these *R* gene products share structural motifs, which indicates that disease resistance to diverse pathogens may operate through similar pathways. For example, leucine-rich repeats (LRRs) are common to most of the *R* genes that have been characterized (Bent et al., 1994; Jones et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994; Grant et al., 1995; Lawrence et al., 1995; Song et al., 1995; Dixon et al., 1996; Anderson et al., 1997; Parker et al., 1997). LRRs have been shown to play a role in protein–protein interactions (Kobe and Deisenhofer, 1994). This fact, along with the common occurrence of LRRs in *R* gene proteins, has led to speculation that LRRs serve as the binding domain for the pathogen-produced elicitor (Bent, 1996; Baker et al., 1997).

Despite recent work in this area, it remains to be proven that LRR-containing *R* gene products function as receptors. In tomato, high-affinity binding sites from intact membranes have been found for an elicitor produced by races of *Cladosporium fulvum* expressing *avr9*, but these binding sites are found in both resistant tomato lines and lines without the corresponding *R* gene *Cf-9* (Kooman-Gersmann et al., 1996). In Arabidopsis, expression of *avrB* and *avrRpt2* within plant leaves induces a defense response exclusively in plants that possess the corresponding *R* genes *RPM1* and *RPS2* (Gopalan et al., 1996; Leister et al., 1996), but a direct interaction has not been reported.

Another class of *R* genes is represented by the *Pto* gene from tomato. The amino acid sequence of Pto reveals a serine/threonine kinase domain, which suggests that protein phosphorylation may play a role in pathogen recognition (Martin et al., 1993). Transient expression of the bacterial

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protein avrPto in plant cells induces a defense response that is dependent on the *Pto* gene, and avrPto and Pto interact in the yeast two-hybrid system (Scofield et al., 1996; Tang et al., 1996). This evidence supports a receptor–ligand model in the case of the Pto kinase.

Pto is a member of a clustered gene family, and one member, *Fen*, confers sensitivity to the insecticide fenthion (Martin et al., 1994; Rommens et al., 1995). Mutations within another gene, *Prf*, affect the function of both *Pto* and *Fen* (Salmeron et al., 1994). Interestingly, *Prf* encodes a protein that is similar to a class of *R* genes that possess LRRs and a nucleotide binding site (NBS) (Salmeron et al., 1996). Thus, for *Pto*-mediated resistance, both NBS/LRR proteins and kinase proteins are required, but specificity is conferred by the kinase component. Whether the involvement of NBS/LRR proteins with kinases is common in *R* gene–mediated pathways is unknown.

In Arabidopsis, accession Columbia (Col-0) possesses the resistance gene *RPS5*, which mediates resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 carrying the heterologous avirulence gene *avrPphB* (formerly called *avrPph3* and originally isolated from the bean pathogen *P. syringae* pv *phaseolicola*) (Simonich and Innes, 1995). Here, we describe the cloning of *RPS5* and the characterization of two *rps5* mutations. The predicted RPS5 protein resembles several previously isolated *R* gene products that contain an NBS and LRRs. Both *rps5* mutations are located within the LRRs. One of the *rps5* mutations affects the function of several other *R* genes that confer resistance to different isolates of *P. s. tomato* and *Peronospora parasitica* (downy mildew). This suggests that at least one region of the LRRs interacts with signal transduction components utilized by multiple *R* gene products.

RESULTS

Isolation of *rps5* Mutants

To identify *rps5* mutants, we inoculated ~16,600 mutagenized Col-0 plants by immersion in a suspension of strain DC3000(*avrPphB*) of *P. s. tomato.* Mutants were identified by the presence of disease symptoms 4 to 5 days after inoculation. From this screen, we isolated two *rps5* mutants derived from separate lots of ethyl methanesulfonate–mutagenized seeds (see Methods). Figure 1 shows that Col *rps5-1* and Col *rps5-2* plants developed disease symptoms of chlorosis and water-soaked lesions after infection with DC3000(*avrPphB*). Wild-type Col-0 plants remained green and healthy. Both mutants were confirmed as susceptible to DC3000(*avrPphB*) by scoring self-progeny.

Genetic analysis of the *rps5* mutants is shown in Table 1. Both mutants were backcrossed to Col-0 plants. All of the F_1 plants were resistant to DC3000(*avrPphB*), indicating that the mutations are recessive. The ratio of resistant-to-susceptible plants was ~3:1 in the F_2 generation, indicating that the susceptible phenotype is caused by a single mutation. To confirm that the mutations were in *RPS5*, we crossed the *rps5* mutants to the accession Landsberg *erecta* (L*er*), which naturally lacks *RPS5* function (Simonich and Innes, 1995). All F_1 and F_2 plants from these crosses were susceptible to DC3000(*avrPphB*). Both mutants were also crossed to each other, and as predicted by the previous result, subsequent generations were susceptible to DC3000(*avrPphB*).

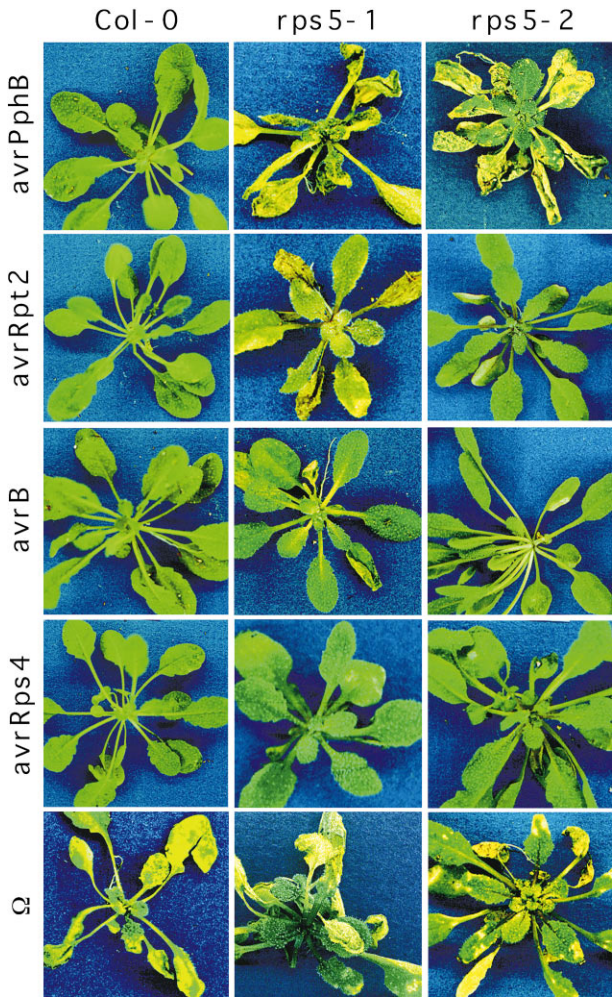

Figure 1. Disease Symptoms Induced by *P. s. tomato* Strains on *rps5* Mutants.

The parental accession Col-0 and the *rps5-1* and *rps5-2* mutants were infected by brief submersion in DC3000 strains carrying the indicated avirulence genes. Ω refers to strain DC3000(*avrB*::Ω), which is a virulent control carrying the *avrB* gene that has been disrupted by the insertion of an Ω fragment. Photographs were taken 5 days after inoculation.

Table 1. Genetic Analysis of *rps5* Mutants[a]

Cross	*avr* Gene Tested	F_1		F_2		
		Resistant	Susceptible	Resistant	Susceptible	χ^{2}[b]
Col-0 × *rps5-1*	*avrPphB*	14	0	81	37	2.5
Col-0 × *rps5-1*	*avrRpt2*	—[c]	—	84	28	0.0
Col-0 × *rps5-2*	*avrPphB*	7	0	58	26	1.6
L*er* × *rps5-1*	*avrPphB*	0	16	0	126	—
L*er* × *rps5-2*	*avrPphB*	0	3	0	14	—
rps5-1 × *rps5-2*	*avrPphB*	0	2	0	40	—

[a] *rps5-1* and *rps5-2* were crossed to the accessions Col-0, which has *RPS5* function, L*er*, which lacks *RPS5* function, and to each other. Plants were scored as resistant or susceptible based on the presence or absence of water-soaked lesions and chlorosis 4 to 5 days after inoculation with *P. s. tomato* carrying the *avr* gene indicated.

[b] χ^2 values for the expected ratio of 3:1 in backcrossed plants ($P > 0.1$ in all cases).

[c] Dashes indicate analysis not performed.

The *rps5-1* Mutation Affects the Function of Multiple Bacterial *R* Genes

In addition to *RPS5*, Col-0 plants possess the *R* genes *RPS2*, *RPM1*, and *RPS4*, which confer resistance to *P. s. tomato* strains carrying *avrRpt2*, *avrB*, or *avrRps4*, respectively (Innes et al., 1993; Kunkel et al., 1993; Hinsch and Staskawicz, 1996). These *avr* genes originally were isolated from *P. syringae* pathovars *tomato*, *glycinea*, and *pisi* but can be expressed heterologously in *P. s. tomato* DC3000. We infected Col-0, Col *rps5-1*, and Col *rps5-2* plants with strain DC3000 carrying each of these *avr* genes. If *RPS5* encodes a receptor that recognizes the elicitor encoded by DC3000(*avrPphB*), then mutations within *RPS5* would not be expected to disrupt the function of these other *R* genes. Col-0 and Col *rps5-2* plants were resistant to all of these pathogen genotypes, as was expected (Figure 1). However, Col *rps5-1* plants developed disease symptoms in response to DC3000 carrying *avrB* or *avrRpt2.* No effect on resistance to DC3000 (*avrRps4*) was observed. In the case of DC3000(*avrB*), lesions developed sporadically and could not be scored consistently, indicating that resistance was only partially compromised. Susceptibility to DC3000(*avrRpt2*) was more easily scored and segregated 3:1 in an F_2 population of backcrossed Col *rps5-1* plants (Table 1). Even so, Col *rps5-1* plants did not appear fully susceptible to DC3000(*avrRpt2*), developing less severe disease symptoms in response to DC3000(*avrRpt2*) than did Col *rps5-1* or Col-0 plants that were infected with a virulent strain of DC3000 (Figure 1).

The increased susceptibility of Col *rps5-1* plants to DC3000(*avrRpt2*) did not appear to be caused by a second-site mutation. We infected F_3 families, which were derived from Col *rps5-1* backcrossed plants, with both DC3000(*avrPphB*) and DC3000(*avrRpt2*). Eight families obtained from DC3000(*avrPphB*)–susceptible F_2 plants developed disease symptoms in response to both bacterial strains, indicating that the phenotypes were caused by the same or closely linked mutations.

Bacterial growth within Col-0, Col *rps5-1*, and Col *rps5-2* plants is quantified in Figure 2. Growth of DC3000(*avrPphB*) was higher in Col *rps5-1* and Col *rps5-2* plants compared with wild-type Col-0 plants and was similar to growth achieved by a virulent strain of *P. s. tomato.* DC3000(*avrRpt2*) and DC3000(*avrB*) consistently grew to higher levels in Col *rps5-1* plants compared with wild-type plants. However, in the majority of trials, this increased growth was not statistically significant. Therefore, although Col *rps5-1* plants develop increased disease symptoms in response to several *P. s. tomato* strains, these symptoms do not reflect a large increase in bacterial growth.

Col *rps5-1* and Col *rps5-2* plants were assayed for their ability to induce a hypersensitive response (HR), a localized response at the site of pathogen infection that is often correlated with disease resistance. The HR is observed as a visible tissue collapse within 24 hr after leaves are infiltrated with avirulent bacteria at a concentration of $\geqslant 10^7$ colony-forming units (cfu) per mL (Whalen et al., 1991). To avoid mistakenly scoring disease symptoms as an HR, *avrPphB* and *avrRpt2* were expressed in a strain of *P. s. glycinea* that does not cause disease in Arabidopsis but, if it contains the appropriate *avr* gene, can induce an HR (Innes et al., 1993). After infiltration of $\sim 2 \times 10^8$ cfu/mL of *P. s. glycinea* carrying *avrPphB*, neither Col *rps5-1* nor Col *rps5-2* plants responded with an HR. However, both Col *rps5-1* and Col *rps5-2* plants retained the ability to induce an HR in response to *P. s. glycinea* carrying *avrRpt2*, supporting the observation that the *rps5-1* mutation only partially compromises resistance conferred by *RPS2.*

The *rps5-1* Mutant Exhibits Decreased Resistance to Several *P. parasitica* Isolates

P. parasitica (a biotrophic oomycete) has emerged as a model eukaryotic parasite of Arabidopsis for characterizing host mutations that affect resistance (Century et al., 1995,

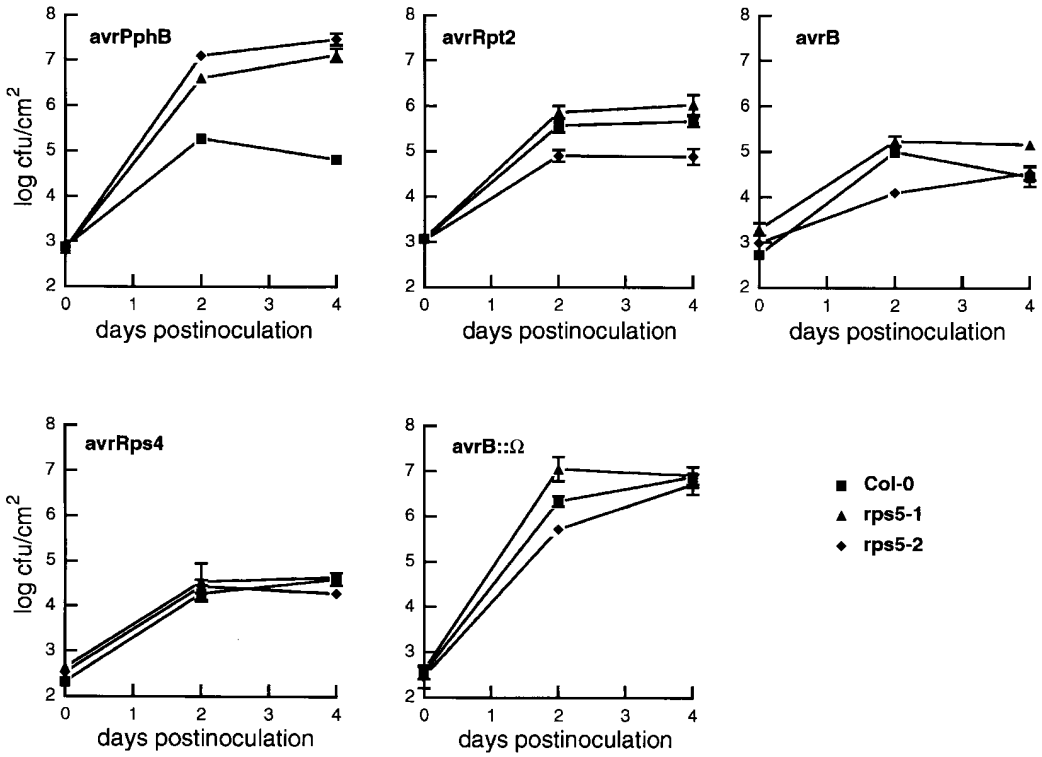

Figure 2. Growth of *P. s. tomato* Strains within Leaves of *rps5* Mutants.

The parental accession Col-0 and the *rps5-1* and *rps5-2* mutants were inoculated by vacuum infiltration with strain DC3000 carrying the indicated avirulence genes. Growth of bacteria within the leaves was monitored over a 4-day time course. Each data point represents the mean ±SE of three samples. Data shown are representative of three independent experiments for DC3000 carrying *avrPphB*, *avrRpt2*, and *avrB*; data are representative of two independent experiments for DC3000 carrying *avrRps4* and *avrB*::Ω.

1997; Parker et al., 1996; Glazebrook et al., 1997; Holub, 1997). We used six isolates of *P. parasitica*, with each being diagnostic for a different wild-type *RPP* (for recognition of *P. parasitica*) gene, to determine whether *rps5* mutations affected resistance to *P. parasitica.* The degree of susceptibility was determined by quantifying asexual sporulation in cotyledons.

Sporulation of five *P. parasitica* isolates was enhanced in Col *rps5-1* plants compared with wild-type Col-0, as shown in Table 2, and contrasted markedly with Col *rps5-2* interactions with the same isolates. The greatest shift toward susceptibility was observed in Col *rps5-1* after inoculation with Emoy2. The shift was from a mean of approximately three sporangiophores per cotyledon in the wild type to >12 in the mutant. Susceptibility of Col *rps5-1* to Hind4, Cand5, Cala2, and Wela3 was enhanced to a lesser degree but nonetheless was statistically significant for each isolate. These results were consistent in three independent experiments. Table 2 shows data for the largest experiment, which included five replications for each combination of accession and isolate. Resistance was not fully compromised in any of the Col *rps5-1* interactions because the number of sporangiophores present was less than that observed in susceptible wild-type interactions (e.g., the accession L*er* infected by Hind4), and necrotic flecks indicative of a resistance response were observed even in the most susceptible interaction between Col *rps5-1* and Emoy2 (data not shown). The enhanced susceptibility in Col *rps5-1* plants was similar to that observed in Col *ndr1-1* plants (Table 2), which were included as a positive control for enhanced susceptibility. Mutations in *NDR1* affect resistance mediated by multiple *R* genes (Century et al., 1995, 1997). In contrast to Col *rps5-1* seedlings, Col *rps5-2* seedlings displayed a statistically significant decrease in resistance to only one isolate, Hind4, and this decrease was small. Resistance to Hiks1 conferred by *RPP7* appeared to be unaffected by either *rps5-1* or *rps5-2.*

The same eight F_3 families that exhibited disease symptoms in response to *P. s. tomato* DC3000(*avrPphB*) and DC3000(*avrRpt2*) were assayed qualitatively in a blind experiment for their response to Emoy2, Cala2, and Hind4. All eight exhibited greater sporulation on their cotyledons than did Col-0 plants (data not shown). These data indicate that disease resistance conferred by several *RPP* loci is affected by the *rps5-1* allele.

Identification of a Candidate *RPS5* Gene

We undertook cloning of the *RPS5* gene to determine its structure and possibly to gain insight into how an *rps5* mutation could affect the function of multiple *R* genes to prokaryotic and eukaryotic pathogens. The genetic map position of *RPS5* was determined using a set of recombinant inbred (RI) lines derived from a cross between accessions Col-0 and L*er* (Simonich and Innes, 1995). This RI population has been used by the Arabidopsis community to map several hundred molecular markers (Anderson, 1996). We found that *RPS5* cosegregated with the marker ATTS0477 in 97 lines. ATTS0477 was of particular interest because it is derived from an expressed sequence tag clone with sequence similarity to cloned *R* genes (GenBank accession number Z17993).

We used ATTS0477 as a hybridization probe to screen a bacterial artificial chromosome (BAC) library of Col-0 genomic DNA (Wang et al., 1996). A BAC clone corresponding to ATTS0477 was not identified from this screen, but we isolated two overlapping BAC clones that contained two sequences that cross-hybridized with ATTS0477 and were tightly linked to *RPS5* (see Methods). One of these two sequences was found to be absent from genomic DNA of accession L*er* when assayed by DNA gel blot hybridization (see Methods). This observation was significant because L*er* lacks *RPS5* function, making the missing sequence a prime candidate for encoding *RPS5.* A similar finding has been reported for the *RPM1* gene of Arabidopsis, which is missing from accessions that lack *RPM1* function (Grant et al., 1995).

Complementation of *rps5* Mutants in Transgenic Plants

Transgenic plants were generated by using Agrobacterium-mediated transformation (Bechtold et al., 1993) to confirm that we had identified *RPS5.* An ~12.4-kb cosmid that contained the putative *RPS5* coding region was placed into a T-DNA binary vector. We transformed this construct into *rps5-1* and *rps5-2* plants. Transgenic (T_1) plants were selected based on kanamycin resistance, transplanted into soil, and allowed to self-fertilize. Five *rps5-1* and four *rps5-2* primary transformants were confirmed as kanamycin resistant in the subsequent (T_2) generation. We tested all nine T_2 families for restoration of *RPS5* function by inoculating them with strain DC3000(*avrPphB*). All nine segregated resistant and susceptible plants, as would be expected for a hemizygous insertion of *RPS5* in the T_1 parent. As shown in Figure 3A, the transgenic plants exhibited the same degree of resistance to the pathogen as did wild-type Col-0 plants. They also remained sus-ceptible to a virulent strain of *P. s. tomato* (data not shown). These results indicated that susceptibility to DC3000(*avrPphB*) was complemented by the 12.4-kb genomic DNA fragment.

We also sought to determine whether the transgene could prevent DC3000(*avrRpt2*) from inducing disease symptoms in the *rps5-1* transgenic plants. We initially tested 15 to 30 plants from each T_2 family. In each of the five families tested, the frequency of symptomless plants was higher than that observed in nontransformed *rps5-1* controls; however, the proportion of resistant to susceptible plants was less than that seen in the same generation of plants infected with DC3000(*avrPphB*) (data not shown). These data suggest that the *avrRpt2*-induced visible phenotype of *rps5-1* might not be fully rescued in the transgenic lines. Two transgenic lines, E29B19 and E29D12, were retested for their response to DC3000(*avrRpt2*) in the T_3 generation. Both lines were derived from independent T_1 plants and, consistent with a T-DNA insertion at a single site, segregated ~3 to 1 for resistance to kanamycin and DC3000(*avrPphB*) in the T_2 generation. In the T_3 generation, all Col E29B19 and Col E29D12 plants were resistant to kanamycin and DC3000(*avrPphB*), indicating they were homozygous for the *RPS5* transgene. After inoculation with DC3000(*avrRpt2*), some Col E29B19 and Col E29D12 plants developed symptoms typical of disease, but the proportion of these plants with symptoms was

Table 2. Asexual Reproduction by Six Isolates of *P. parasitica* in Wild-Type and Mutant Lines of Arabidopsis[a]

Arabidopsis Line	*P. parasitica* Isolate[b]																	
	Emoy2 (*RPP4*)			Hind4 (*RPP9*)			Cand5 (Not Mapped)			Cala2 (*RPP2*)			Wela3 (*RPP6*)			Hiks1 (*RPP7*)		
	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*
Col-0	2.9	0.6	34	1.6	0.4	69	0.9	0.3	51	0.0	0.0	57	0.0	0.0	60	0.0	0.0	45
Col *rps5-1*	12.1	0.8	30	4.7	0.7	41	4.3	0.7	42	0.3	0.1	32	1.2	0.4	48	0.0	0.0	47
Col *rps5-2*	3.7	0.4	43	3.3	0.6	50	1.0	0.2	62	0.1	0.1	48	0.0	0.0	54	0.0	0.0	50
Col *ndr1*	9.8	0.7	63	2.5	0.5	54	19.2	0.4	38	0.0	0.0	50	0.7	0.1	53	0.0	0.0	39
L*er*	0.0	0.0	37	20.0	0.0	35	0.0	0.0	39	19.1	0.2	35	15.6	0.6	51	0.0	0.0	47

[a] Measured as the mean number of sporangiophores per cotyledon; maximum of 20 counted.

[b] *RPP* gene in Col-0 conferring resistance to each *P. parasitica* isolate (see Holub, 1997). SEM, standard error of the mean; *n*, number of seedlings inoculated and distributed among five replications.

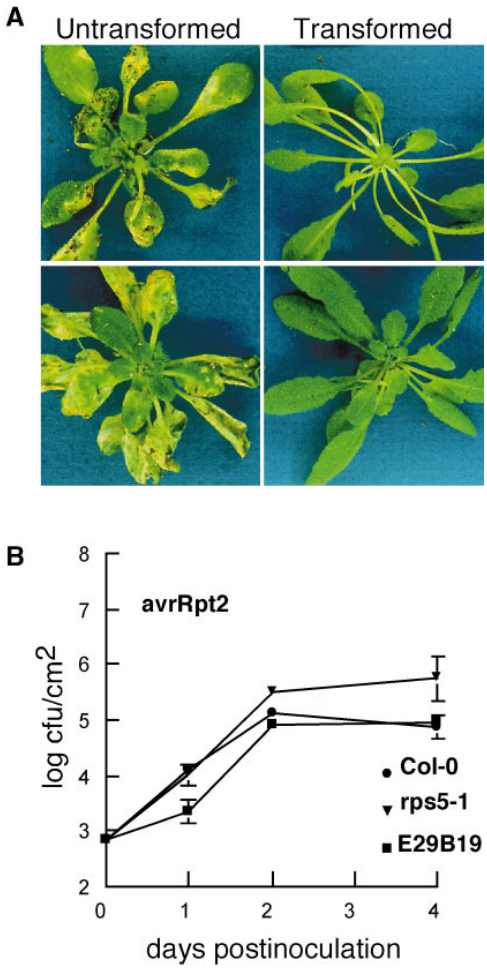

Figure 3. Complementation of *rps5* Mutations.

(A) Restoration of *avrPphB* recognition. The *rps5-1* and *rps5-2* mutants were transformed with a 12.4-kb genomic fragment containing the wild-type *RPS5* gene from Arabidopsis accession Col-0. Transformed and untransformed lines were infected by brief submersion in *P. s. tomato* DC3000 carrying *avrPphB.* Photographs were taken 5 days after inoculation.

(B) Growth of *P. s. tomato* (*avrRpt2*) within transgenic *rps5-1* plants. Col-0, Col *rps5-1*, and Col E29B19 plants, which are *rps5-1* mutants homozygous for an *RPS5* transgene, were inoculated by vacuum infiltration with strain DC3000 carrying *avrRpt2.* Growth of bacteria within the leaves was monitored over a 4-day time course. Each data point represents the mean ±SE of three samples.

less than the proportion among the Col *rps5-1* control plants (data not shown).

The above results suggest that the 12.4-kb clone at least partially suppressed the *avrRpt2*-induced visible phenotype observed in Col *rps5-1* plants. Bacterial growth of DC3000(*avrRpt2*) within Col E29B19 plants was also assayed quantitatively. As shown in Figure 3B, the level of growth within Col E29B19 plants was indistinguishable from that seen in wild-type Col-0 plants. Bacterial growth within *rps5-1* plants was higher at 2 and 4 days after inoculation than in either wild-type Col-0 or Col E29B19 plants, and increased growth was statistically significant on day 2.

We also assayed the transgenic lines for restoration of resistance to *P. parasitica.* Table 3 shows the mean number of sporangiophores produced by three *P. parasitica* isolates in Col E29B19 and Col E29D12 T_3 plants. Resistance to isolate Cala2 was fully restored in both transgenic lines. Resistance to Emoy2 was also fully recovered in Col E29D12 plants. On Col E29B19 plants, however, the number of sporangiophores produced by Emoy2 was intermediate between wild-type Col-0 and Col *rps5-1* plants. When the isolate Emwa1 was tested, this intermediate phenotype was seen in both transgenic plant lines. These data indicate that the suppressive effect of the *rps5-1* mutation on *RPP4* is only partially corrected by the wild-type *RPS5* gene.

Structure of the Putative *RPS5* Gene

We sequenced a 7.1-kb genomic region that contained the putative *RPS5* gene and the adjacent *R* gene–like sequence, which we designated *RFL1* (for *RPS5*-like). Two large open reading frames (ORFs) that lacked introns were identified and are shown in Figure 4. Both ORFs were oriented in the same direction and were separated by 1408 bp. Both ORFs were found to be present on the 12.4-kb cosmid used for complementation. The putative *RPS5* gene that was absent from accession L*er* corresponded to the downstream ORF. This ORF was confirmed to be *RPS5* by sequence analysis of the *rps5-1* and *rps5-2* alleles (see below). The bases flanking the first ATG of the *RPS5* ORF (CAGAATGGG) are consistent with the consensus sequence for translation initiation in plants (Lutcke et al., 1987), and an in-frame stop

Table 3. Asexual Reproduction by *P. parasitica* in Transgenic *rps5-1* Plants[a]

Arabidopis Line	*P. parasitica* Isolate[b]								
	Emoy2 (*RPP4*)			Emwal (*RPP4*)			Cala2 (*RPP2*)		
	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*
Col-0	1.3	0.2	72	0.4	0.1	54	0.2	0.1	76
Col *rps5-1*	4.6	0.3	81	5.3	0.4	75	0.9	0.1	71
Col E29B19	2.2	0.2	83	2.1	0.2	77	0.2	0.1	86
Col E29D12	0.9	0.1	113	1.3	0.2	72	0.2	0.1	83

[a] Measured as the mean number of sporangiophores per cotyledon; maximum of 20 counted.

[b] *RPP* gene in Col-0 conferring resistance to each *P. parasitica* isolate (see Holub, 1997). SEM, standard error of the mean; *n*, number of seedlings inoculated.

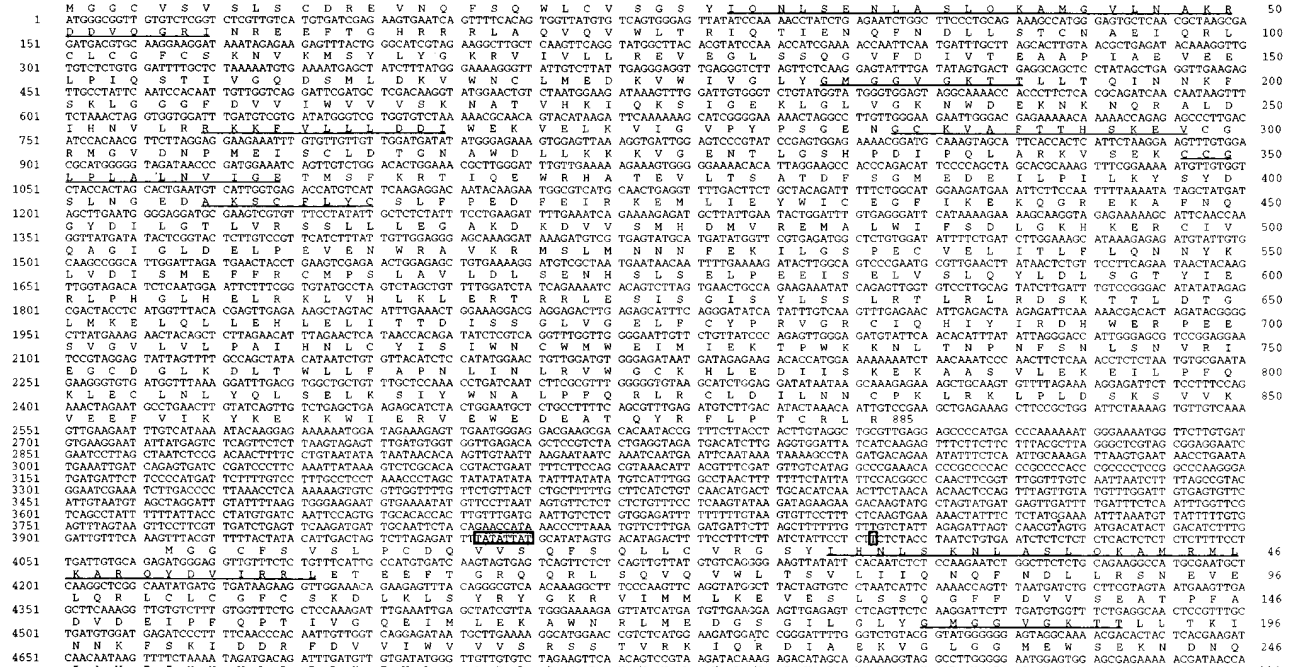

Figure 4. *RPS5* and *RFL1* Encode *R* Gene Products of the NBS/LRR Superfamily and Are Arranged as a Tandem Repeat.

Shown is the DNA sequence of a 6.7-kb genomic region encoding the *RFL1* (top ORF) and *RPS5* (bottom ORF) genes. Predicted translation products are given above the DNA sequence. Underlined amino acids indicate domains that are conserved within the NBS/LRR family. Starting at the N terminus of each protein, these are a putative leucine zipper; a putative NBS consisting of a P loop, a kinase-2a domain, and a kinase-3a domain; and two additional conserved domains of unknown function, as defined by Grant et al. (1995). The LRR region is featured in Figures 5 and 6. The amino acids altered by the *rps5-1* (amino acid position 572) and *rps5-2* (amino acid position 799) mutations are boxed. A potential TATA box upstream of the *RPS5* gene is also boxed, as is the most upstream nucleotide identified by 5′ RACE.

codon (TAG) is present 90 bases upstream of the start codon. A typical TATA box sequence (TATATTAT) is present 111 bases upstream of the start codon. We amplified cDNA from total leaf RNA derived from wild-type Col-0 and utilized rapid amplification of cDNA ends (RACE) technology to define the approximate 5′ end of the *RPS5* transcript. Analysis of four independent RACE clones revealed the same 5′ end 61 bases upstream of the first ATG in the *RPS5* ORF, indicating that transcription starts near this region (Figure 4).

The deduced amino acid sequences that correspond to the *RPS5* and *RFL1* ORFs are shown in Figure 4. The two proteins are similar to each other (66% identical; 77% similar). Among *R* genes with a known function, the RPS5 sequence most closely resembles the amino acid sequence from RPS2 (36% identical; 57% similar) and RPM1 (23% identical; 49% similar), whose genes also lack introns. Sequence comparison identified many motifs seen in previously cloned *R* genes. The RPS5 protein contains a putative NBS composed of kinase-1a (or P-loop; amino acids 183 to 191), kinase-2a (amino acids 258 to 267), and putative kinase-3a (amino acids 285 to 298) domains (Saraste et al., 1990; Traut, 1994; Grant et al., 1995). The C-terminal region of *RPS5* is composed of 13 imperfect LRRs (Kobe and Deisenhofer, 1994), as shown in Figure 5, beginning at amino

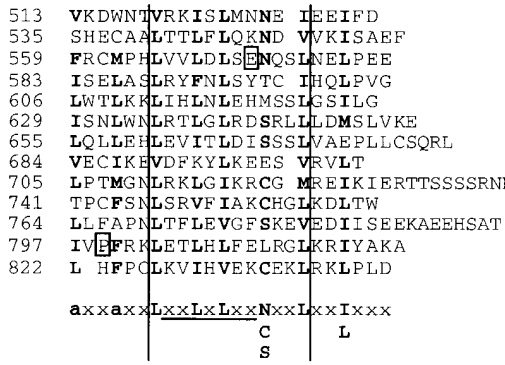

Figure 5. LRR Region of RPS5.

The amino acid sequence for the RPS5 LRRs is shown. The consensus sequence for a RPS5 LRR is given at the bottom, and the vertical bars demarcate the conserved consensus region present in plant, animal, and fungal LRR proteins (Jones and Jones, 1997). The bar under the consensus LRR indicates the putative β strand/β turn region postulated to be involved in ligand binding. An x represents an arbitrary amino acid residue, and a boldface, lowercase **a** represents a hydrophobic (L, I, M, V, or F) residue. Single residues shown in the consensus comprised >50% of the residues at that position. Multiple residues at a position in the consensus together comprised >50% of the residues at that position. Residues that match the consensus sequence, allowing hydrophobic residues to substitute for each other, are shown in boldface. The amino acids altered in *rps5-1* (E572K) and *rps5-2* (P799S) are boxed.

acid 513. A potential leucine zipper is present at amino acids 29 through 57 (Alber, 1992). An additional two uncharacterized motifs are present (amino acids 348 to 360 and 408 to 415) that are well conserved in products of previously isolated *R* genes (Grant et al., 1995; Staskawicz et al., 1995). Analogous motifs are present in the RFL1 sequence (Figure 4).

The *rps5* Mutations Are Contained within LRRs

To verify that we had identified *RPS5* and to gain insight into the nature of the *rps5-1* mutation, we sequenced both the *rps5-1* and *rps5-2* alleles. Primers were designed from the coding region of the putative *RPS5* gene and used to amplify overlapping fragments from *rps5-1* and *rps5-2* genomic DNA. Four independent polymerase chain reaction (PCR) amplifications were pooled for each primer set and were sequenced directly. We determined that mutations were present in both *rps5* mutant plants when compared with DNA from wild-type Col-0. Both *rps5* mutations contained single base pair changes that altered the amino acid sequence. The *rps5-1* mutation caused a G-to-A transition, which results in a glutamate-to-lysine change at amino acid 572, whereas the *rps5-2* mutation caused a C-to-T transition, which leads to a proline-to-serine change at amino acid 799. Both mutations are located in the LRR region (Figure 5).

Figure 6 shows an alignment of the amino acid sequences from the LRR region between RPS5, RFL1, RPS2, and RPM1. The *rps5-1* mutation is contained in the third LRR, which is the most highly conserved of the 13 LRRs present in these proteins (35 to 79% identity to RPS5). This observation suggests that this region may serve a related function in each of these proteins. The *rps5-2* mutation is in the 12th LRR, a region with less sequence identity among the *R* genes.

DISCUSSION

We have cloned the resistance gene *RPS5* and characterized two mutations within the *RPS5* gene. *RPS5* confers re-

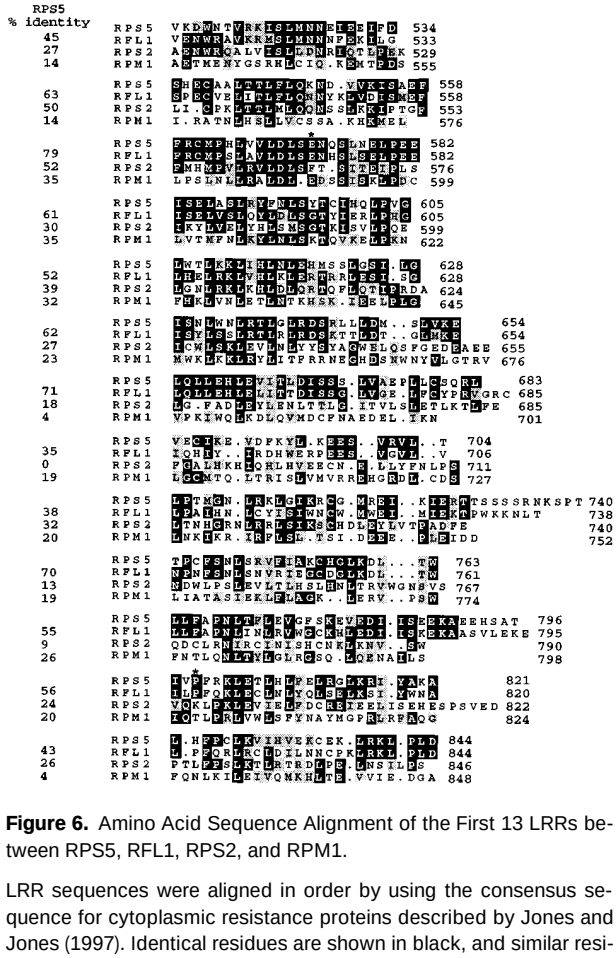

Figure 6. Amino Acid Sequence Alignment of the First 13 LRRs between RPS5, RFL1, RPS2, and RPM1.

LRR sequences were aligned in order by using the consensus sequence for cytoplasmic resistance proteins described by Jones and Jones (1997). Identical residues are shown in black, and similar residues are shown in gray. Sequence gaps are indicated with dots. An asterisk is placed above the amino acids of RPS5 altered in the *rps5-1* and *rps5-2* alleles. Shown to the left of the sequence alignment is the percentage of identity between RPS5 and RFL1, RPS2, and RPM1 for each repeat.

sistance to *P. s. tomato* strains carrying the avirulence gene *avrPphB* (Simonich and Innes, 1995). The *rps5-1* mutation not only disrupted *RPS5* function but also partially affected the function of genes that mediate resistance to *P. s. tomato* strains carrying other avirulence genes as well as to several isolates of *P. parasitica* (Figure 1 and Table 2). In contrast, the *rps5-2* mutation had little to no effect on resistance to *P. s. tomato* and *P. parasitica* strains other than the *P. s. tomato* DC3000(*avrPphB*). This difference between *rps5-1* and *rps5-2* suggests that the mutations reside in regions of the *RPS5* gene that perform different functions in a disease resistance pathway.

With the exception of race-specific resistance mediated by *RPS5*, the *rps5-1* mutation did not completely abolish resistance conferred by the *R* genes tested (Figure 1 and Table 2). This suggests that the *rps5-1* mutation acts either by delaying pathogen recognition or by interfering with a subset of events that occur after pathogen recognition. For *P. parasitica*, increased development of disease symptoms correlated with increased sporulation on Col *rps5-1* cotyledons (Table 2). Disease symptoms induced by *P. s. tomato* carrying *avrRpt2* or *avrB* also correlated with increased pathogen growth, but this increased growth was not always statistically significant (Figure 2). The oomycete sporulation assay may be a more sensitive method to detect partial loss of resistance than are bacterial growth assays.

The degree of symptoms exhibited among Col *rps5-1* plants varied depending on which *P. s. tomato* strain or *P. parasitica* isolate was being tested (Figure 1 and Table 2). The different phenotypes observed could be related to functional differences among the *R* genes. Different *R* genes can exhibit differences in the strength or timing of the hypersensitive resistance response (Hammond-Kosack and Jones, 1994; Century et al., 1995) and differences in the secondary pathways induced subsequent to pathogen recognition (Reuber and Ausubel, 1996). The mutant rps5-1 protein may affect a pathway or a factor that is more critical to some *R* genes than to others.

Structural Properties of RPS5

RPS5 belongs to the NBS/LRR class of plant *R* genes (reviewed in Bent, 1996; Baker et al., 1997; Hammond-Kosack and Jones, 1997). Of genes with known function, *RPS5* encodes a protein most similar to RPS2 and RPM1 that, in addition to the other conserved motifs, contains a putative leucine zipper near the N terminus. *RPS2* and *RPM1* also lack introns and are thought to be intracellularly localized (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995). Leucine zipper domains have been shown to facilitate protein–protein interactions, including formation of homodimers and heterodimers for some proteins (Alber, 1992). The presence of leucine zipper domains in a subset of NBS/LRR proteins may indicate that the leucine zipper plays a specific role in signal transduction for these proteins.

The LRRs consist of a repeated motif of ~24 amino acids, contain leucines or other hydrophobic residues at regular intervals, and have been shown to mediate protein–protein interactions (Kobe and Deisenhofer, 1994). Alignment of the LRRs from numerous plant, animal, and fungal proteins has revealed a conserved core motif of LxxLxLxx(N/C/T)xL within each LRR where an x represents an arbitrary amino acid sequence (Jones and Jones, 1997; Figure 5). Based on comparison with the porcine ribonuclease inhibitor protein, for which the crystal structure has been determined, the central xxLxLxx portions of each repeat are believed to align, forming a parallel β sheet flanked by parallel β turns. This structure forms a relatively flat surface in which the leucines are buried in the center of the protein and the adjacent residues are exposed to the solvent (Kobe and Deisenhofer, 1994; Jones and Jones, 1997). For the porcine ribonuclease inhibitor protein, 20 of the 28 contacts with its ligand (ribonuclease A) occur on this surface (Kobe and Deisenhofer, 1995). Although the LRRs of NBS/LRR proteins are more degenerate than those in the porcine ribonuclease inhibitor protein, the relatively high conservation of the core motif suggests that it too may form a solvent-exposed surface.

Of the four known missense mutations within the LRR domains of the RPM1 and RPS2 *R* gene–encoded proteins, all occur in the xxLxLxx motif (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995), suggesting that like the porcine ribonuclease inhibitor, this surface participates in ligand binding. Consistent with this hypothesis, the glutamate residue altered by the *rps5-1* mutation also lies within the xxLxLxx motif (Figure 5).

Alignment of the LRR regions of RPS5, RFL1, RPS2, and RPM1 revealed that RFL1 and RPM1 also have a glutamate residue at the position affected by *rps5-1* (Figure 6). However, in RPS2, this position is occupied by a hydrophobic phenylalanine residue, a significant amino acid change. The *rps5-1* mutation causes substitution of a lysine at this position, changing a negative charge to a positive charge. With regard to affecting the function of other *R* genes, the change to a positively charged residue may be the critical factor.

The *rps5-2* mutation causes a proline-to-serine change within the 12th LRR (Figure 5). This proline is located outside of the conserved LRR core motif. There are 11 prolines located in the RPS5 LRR region, and all are positioned outside the conserved core domain (Figure 5). Jones and Jones (1997) have speculated that prolines, which cause kinks in the peptide backbone, may function in positioning the conserved core motifs. The *rps5-2* mutation may thus be disrupting the general structure of a ligand binding surface of RPS5.

Putative Roles of RPS5 in Pathogen Resistance

The *rps5-1* mutation is present in a region of the LRRs that is relatively well conserved between RPS5 and RFL1 and RPS2 (79 and 52% amino acid identity; Figure 6). This region also showed the most similarity to RPM1 (35%), but conservation

between RPS5 and RPM1 was weak throughout the LRRs. The *rps5-2* mutation is present in a region that showed less sequence identity with RFL1, RPS2, and RPM1 (56, 24, and 20% amino acid identity). We speculate that the region around the third LRR serves a related function in RPS5, RFL1, RPS2, and possibly RPM1. The less conserved, more C-terminal LRRs may represent the region responsible for specificity to particular avirulence determinants. A similar idea was proposed for the *R* genes *Cf-9* and *Cf-2.* These genes encode membrane-anchored proteins with extracytoplasmic LRRs of 27 and 38 repeats, respectively (Jones et al., 1994; Dixon et al., 1996). The C-terminal LRRs share high similarity with each other, and it has been suggested that the conserved regions could interact with similar components of a signal transduction pathway (Jones and Jones, 1997; Thomas et al., 1997). That specific parts of the LRR region of RPS5 may fulfill different functions is supported by the different pathogen responses observed between *rps5-1* and *rps5-2* plants.

Homozygous *rps5-1* plants have lost the ability to induce resistance to *P. s. tomato* carrying *avrPphB* (Table 1 and Figures 1 and 2), and this susceptibility is fully corrected by an *RPS5* transgene (Figure 3A). The *RPS5* transgene also restored resistance to *P. s. tomato* strains carrying *avrRpt2* in *rps5-1* mutant plants, as assayed by bacterial growth in leaves (Figure 3B). However, inconsistent with full complementation, some transgenic *rps5-1* plants still developed disease symptoms, indicating that the mutant rps5-1 protein may still interfere with resistance specified by *RPS2.* Similar results were obtained for resistance to *P. parasitica.* The *RPS5* transgene did not fully restore resistance to the Emwa1 isolate in two transgenic lines and did not fully restore resistance to the Emoy2 isolate in one of the transgenic lines (Table 3).

To explain the behavior of the *rps5-1* mutation in the transgenic lines, we propose that the *rps5-1*–encoded protein titrates out a component used by multiple *R* gene–mediated pathways. Common motifs shared among *R* gene proteins imply that a common signal transduction pathway may exist. Consistent with this hypothesis, a number of mutants have been identified that affect resistance conferred by multiple *R* genes (Hammond-Kosack and Jones, 1996; Baker et al., 1997). The Arabidopsis mutation *ndr1* suppresses resistance conferred by *RPS2*, *RPM1*, *RPS5*, and several *RPP* loci in the Arabidopsis accession Col-0 (Century et al., 1995, 1997). Col *rps5-1* plants are affected to a similar degree in response to *P. parasitica*, as are Col *ndr1* plants (Table 2). In the Wassilewskija (Ws-0) accession, the *eds1* mutation affects several *RPP* specificities (Parker et al., 1996). In addition, we have identified and are currently characterizing mutations in two genes that compromise the function of multiple *R* genes (R.F. Warren and R.W. Innes, unpublished results). Any of these four genes could encode proteins that may be titrated by the *rps5-1*–encoded protein. Such a protein may exhibit different binding affinities to different *R* gene–encoded proteins, which would explain the varying effects of both the *rps5-1* mutation and the wild-type transgene on the function of different *R* genes.

The failure to detect increased susceptibility in *RPS5*/*rps5-1* heterozygotes and the partial recovery of resistance specified by *R* genes other than *RPS5* in *rps5-1* transgenic plants could be due to competition between wild-type and mutant RPS5 proteins. For example, if RPS5 forms homodimers, which is consistent with the presence of the leucine zipper motif, then expression of the wild-type allele in the *rps5-1* background should result in formation of heterodimers of mutant and wild-type protein. Such heterodimers might not sequester the shared factor as effectively as rps5-1 homodimers.

Given the proposed role of LRRs and the presence of the *rps5-1* mutation in a relatively conserved area, it seems most likely that this mutation increases binding affinity for a protein that interacts with this region. However, we have not eliminated the possibility that the *rps5-1* mutation increases protein stability, allowing the mutant protein to sequester more of a factor shared among *R* gene–mediated signal transduction pathways.

There are alternatives to this titration model. For example, in addition to recognizing an *avrPphB*-derived elicitor, *RPS5* could weakly recognize other *avr*-based signals. In this case, recognition of these signals is retained in *rps5-2* plants but is abolished in *rps5-1* plants. However, this model and related models still must explain partial complementation exhibited in transgenic plants, the segregation of *rps5-1* as a single recessive allele, and the failure to detect decreased pathogen resistance in L*er* plants, which lack the *RPS5* gene. The *R* genes whose functions are affected by *rps5-1* have been mapped to singular chromosome locations distinct from *RPS5.* To distinguish between the titration model and possible alternatives, we plan to overexpress the *rps5-1* allele in a wild-type background. If the titration model is correct, such overexpression should suppress multiple *R* gene pathways, whereas it should have no effect if *rps5-1* is a simple loss-of-function mutant.

METHODS

Pseudomonas Strains and Peronospora Isolates

Pseudomonas syringae strains were cultured as described previously (Innes et al., 1993). *P. syringae* pv *tomato* strains carrying *avrB*, *avrB*::Ω, *avrRpt2*, *avrRps4*, and *avrPphB* have been described previously (Innes et al., 1993; Simonich and Innes, 1995; Hinsch and Staskawicz, 1996). The *Peronospora parasitica* isolates and their cultivation have also been described previously (Dangl et al., 1992; Holub et al., 1994).

Growth of Plants, Plant Inoculations, and Bacterial Growth Curves

Growth conditions for *Arabidopsis thaliana* were as described previously (Bisgrove et al., 1994). Mutagenized seeds (M_2 generation) were obtained from M. Estelle (Indiana University, Bloomington, IN; ethyl methanesulfonate–mutagenized and γ-irradiated seeds). In all

cases, mutagenesis was performed with seeds (M_1 generation), and plants were allowed to self-fertilize. Seeds from ~500 M_1 plants were pooled to generate bulked M_2 seed lots. Thirty-two lots were screened to identify the *rps5* mutants. Plants were inoculated by dipping whole rosettes in a suspension of $\sim 2 \times 10^8$ colony-forming units (cfu) of *P. s. tomato* per mL, as previously described (Innes et al., 1993). Genotypes of putative mutants were confirmed as being Columbia (Col-0) and not a contaminating susceptible genotype using several microsatellite and cleaved amplified polymorphic sequence markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). To monitor bacterial growth in Arabidopsis leaves, we inoculated plants by vacuum infiltration of 5×10^5 cfu/mL of suspension of *P. s. tomato*, as described by Whalen et al. (1991). The surfactant Silwet L-77 (OSi Specialties, Inc., Danbury, CT) was added at a concentration of 0.001%. Samples were removed from rosette leaves, macerated, diluted, and plated on selective medium, as described previously (Bisgrove et al., 1994). Colonies were counted 48 hr later. Resistance of Arabidopsis accessions to *P. parasitica* was assayed by inoculating seedling cotyledons, as described previously (Dangl et al., 1992; Holub et al., 1994). A minimum of 30 seedlings distributed among five replications was used per plant genotype per *P. parasitica* isolate combination in all experiments.

Genetic Analysis

Crosses were performed by hand-emasculating flowers before anther dehiscence and then brushing donor pollen over the stigmas. F_1, F_2, and F_3 plants were scored for disease phenotypes by using the dip assay. Seeds were collected from individual selfed F_1 and F_2 plants to generate plants for the next generation.

DNA and RNA Methods and Cloning

The isolation of bacterial artificial chromosome (BAC) clones that cross-hybridized with ATTS0477 has been described previously (Wang et al., 1996). The *RPS5* and *RFL1* genes were present on BAC clones dBAC24D20 and dBAC5D5 and were identified as separate hybridizing restriction fragments on a DNA gel blot probed with ATTS0477. *RPS5* and *RFL1* sequences were gel purified for use as hybridization probes. Mapping of the *RFL1* and *RPS5* sequences was accomplished by hybridization of *RFL1* and *RPS5* probes with DNA gel blots of yeast artificial chromosome (YAC) clones. YACs that map to the RPS5 region (http://cbil.humgen.upenn.edu/~atgc/physical_ mapping/ch1_ptl.html) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). *RFL1*, *RPS5*, and ATTS0477 probes hybridized with different-sized fragments of HindIII-digested DNA from the YACs CIC12H10 and CIC9G11. The *RPS5* probe detected only a single 4.1-kb HindIII fragment in Col-0 and no band in Landsberg *erecta* (L*er*). Standard protocols were used for restriction digests, DNA gel blotting, and probe preparation (Ausubel et al., 1987; Sambrook et al., 1989). Large-scale genomic plant DNA preparations were performed as described previously (Ashfield et al., 1998).

Agrobacterium-Mediated Transformation

Transgenic plants were constructed by infiltrating Arabidopsis inflorescences with *Agrobacterium tumefaciens* GV3101 carrying the transgene of interest by methods previously described (Bechtold et al., 1993; Bent et al., 1994). A fragment containing *RPS5* was subcloned from dBAC5D5. To subclone *RPS5* into the binary vector pCLD04541 (Bancroft et al., 1997), dBAC5D5 was partially digested with Sau3A, treated with calf intestine alkaline phosphatase to prevent ligation of noncontiguous fragments, ligated with a BamHI-digested vector, and packaged using a Gigapack Gold III kit (Stratagene, La Jolla, CA). A clone containing *RPS5* was identified through restriction analysis and confirmed as full length by polymerase chain reaction (PCR).

DNA Sequencing of *RPS5* and *RFL1*

DNA restriction fragments from dBAC24D20 and dBAC5D5 were subcloned into the pBluescript KS+ vector (Stratagene) and propagated in *Escherichia coli* DH5α. DNA was isolated using a plasmid kit following the manufacturer's protocol (Qiagen Inc., Chatsworth, CA). Sequencing was performed using a SequiTherm long read cycle sequencing kit (Epicentre Technologies, Madison, WI) with IRD41 end-labeled T3, T7, or M13 reverse sequencing primers (LiCor, Inc., Lincoln, NE) on a LiCor 4000L DNA sequencer. Additional sequencing was performed using an ABI dye terminator FS kit protocol (Perkin-Elmer, Foster City, CA) on an ABI Prism 377 DNA sequencer. Evaluation of sequencing data and construction of sequence contigs were performed with the Sequencher software package for the Power Macintosh (GeneCodes Corporation, Ann Arbor, MI). We amplified cDNA by using a Marathon cDNA amplification kit (Clonetech, Palo Alto, CA) and performed 5′ rapid amplification of cDNA ends (RACE) following the manufacturer's protocol. RACE products were generated using an adapter primer from the kit and an *RPS5* gene–specific primer. These were subcloned into pBluescript KS+ (Stratagene), and four clones were sequenced to define the 5′ end of the transcript. Homology searches of the GenBank database were performed using the BLAST2 algorithm (Altschul et al., 1997), and alignment of sequences was performed using the GAP program of the Genetics Computer Group (Madison, WI) Wisconsin Package version 9.1. The sequence shown in Figure 4 has been submitted to GenBank (accession number AF074916).

PCR-Based Sequencing of *rps5* Alleles

The coding sequence for the *rps5* alleles was amplified as five separate overlapping fragments from genomic DNA by using PCR. Four of the primer pairs included the T7 sequence at the 5′ end of the primer and the M13 reverse sequence at the 5′ end of the other, allowing direct sequencing using T7 and M13 reverse sequencing primers. Pooled products of four independent PCR reactions were purified by filtration (Ultrafree-MC filter unit, 30,000 D cutoff; Millipore, Bedford, MA), and 100 to 200 ng of DNA was used as template for sequencing with the LiCor sequencer. Mutations were confirmed on both strands. A fifth primer set that lacked the M13 reverse and T7 5′ extensions was also used. The PCR product amplified by this primer pair was purified, and 100 to 200 ng was used as template and sequenced using the dye terminator protocol on an ABI sequencer.

ACKNOWLEDGMENTS

We thank Dr. Fred Ausubel and Eliana Drenkard for sharing mapping data for marker ATTS0477 and the Arabidopsis Biological Resource Center at Ohio State University for providing expressed sequence

tag and YAC clones. We also thank Dr. Mark Estelle for providing mutagenized Arabidopsis seed, Figen Mert (Ph.D. student with E.H.) for confirmation of mutant responses to *P. parasitica*, and John Danzer and Sandra Szerszen for technical assistance. Research in Indiana was supported by Grant No. R01 GM46451 from the Institute of General Medical Sciences of the National Institutes of Health to R.W.I. Research at Horticultural Research International was supported by core funding to E.H. from the Biotechnology and Biological Systems Research Council.

Received April 22, 1998; accepted July 10, 1998.

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