Identification of Three Putative Signal Transduction Genes Involved in *R* Gene-Specified Disease Resistance in Arabidopsis

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ABSTRACT

The *RPS5* disease resistance gene of Arabidopsis mediates recognition of *Pseudomonas syringae* strains that possess the avirulence gene *avrPphB*. By screening for loss of *RPS5*-specified resistance, we identified five *pbs* (*avrPphB* susceptible) mutants that represent three different genes. Mutations in *PBS1* completely blocked *RPS5*-mediated resistance, but had little to no effect on resistance specified by other disease resistance genes, suggesting that PBS1 facilitates recognition of the avrPphB protein. The *pbs2* mutation dramatically reduced resistance mediated by the *RPS5* and *RPM1* resistance genes, but had no detectable effect on resistance mediated by *RPS4* and had an intermediate effect on *RPS2*-mediated resistance. The *pbs2* mutation also had varying effects on resistance mediated by seven different *RPP* (recognition of *Peronospora parasitica*) genes. These data indicate that the PBS2 protein functions in a pathway that is important only to a subset of disease-resistance genes. The *pbs3* mutation partially suppressed all four *P. syringae*-resistance genes (*RPS5*, *RPM1*, *RPS2*, and *RPS4*), and it had weak-to-intermediate effects on the *RPP* genes. In addition, the *pbs3* mutant allowed higher bacterial growth in response to a virulent strain of *P. syringae*, indicating that the *PBS3* gene product functions in a pathway involved in restricting the spread of both virulent and avirulent pathogens. The *pbs* mutations are recessive and have been mapped to chromosomes I (*pbs2*) and V (*pbs1* and *pbs3*).

PATHOGEN resistance in plants is often characterized by a gene-for-gene relationship requiring a specific resistance (*R*) gene from the plant and a corresponding avirulence (*avr*) gene from the pathogen (Flor 1971). The presence of the appropriate *R-avr* gene pair results in host resistance, whereas the absence or inactivation of either member of the gene pair results in susceptibility of the host to the pathogen. Although the molecular mechanisms are still unknown, *R* genes mediate specific recognition of pathogens, presumably by some sort of receptor-ligand interaction (Gabriel and Rolfe 1990).

After initial pathogen recognition, signaling events that result in the activation of plant defenses and the limitation of pathogen growth are triggered. These defense responses are often correlated with rapid, localized necrosis at the site of infection (hypersensitive response), and they include an oxidative burst, cell wall fortification, production of antimicrobial compounds (phytoalexins), and the accumulation of pathogenesis-related proteins (Hammond-Kosack and Jones 1996). *R* genes that mediate resistance to bacterial, fungal, oomycete, viral, and nematode pathogens have been cloned from several plant species (reviewed in Bent 1996; Baker *et al.* 1997; Jones and Jones 1997; Ellis and Jones 1998). Structural motifs are shared among many of these *R* gene proteins, indicating that disease resistance to diverse pathogens may operate through similar molecular pathways. However, components of these pathways and their function remain largely undefined.

To identify potential signal transduction components used by *R* genes, we and others have screened mutagenized plants for loss of resistance to specific pathogens (see reviews by Hammond-Kosack and Jones 1996; Kunkel 1996; Baker *et al.* 1997; Innes 1998). By design, these screens also identify mutations within *R* genes. In fact, the isolation of *R* gene mutants appears to be much more common than the identification of mutants involved in *R* gene-mediated signal transduction pathways (Innes 1998). This may indicate that some pathway components are redundant or required for viability. Additionally, these pathways may be branched such that a particular mutation abolishes only a subset of defense responses. Such mutants may have been overlooked because pathogen resistance was not completely compromised. To date, only a small number of putative *R* gene signal transduction mutants have been identified from genetic screens for loss of pathogen resistance. These include mutants in barley (*rar1* and *rar2*; Torp and Jorgensen 1986; Jorgensen 1988; Freialdenhoven *et al.* 1994), tomato (*rcr1*, *rcr2*, and *prf*); (Hammond-Kosack *et al.* 1994; Salmeron *et al.* 1994), and Arabi-

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dopsis (*ndr1* and *eds1*); (Century *et al.* 1995; Parker *et al.* 1996).

Most of these mutations affect the function of a subset of *R* genes tested. The *rar1* and *rar2* mutations reduce resistance conferred by several powdery mildew-resistance genes (Freialdenhoven *et al.* 1994; Jorgensen 1996). The *ndr1* and *eds1* mutations affect resistance against both bacterial and oomycete pathogens (Century *et al.* 1995, 1997; Parker *et al.* 1996; Aarts *et al.* 1998). Interestingly, *R* genes that are strongly suppressed by one of these Arabidopsis mutations are not greatly affected by the other mutation, indicating that *NDR1* and *EDS1* may be critical for different signaling pathways (Aarts *et al.* 1998). The *rcr1*, *rcr2*, and *prf* mutations are reported to affect pathogen resistance conferred by single *R* genes, but their effect on the function of other *R* genes has not been tested extensively (Hammond-Kosack *et al.* 1994; Salmeron *et al.* 1994).

Although the identified mutations reduce resistance conferred by specific *R* genes, most do not eliminate all defense responses. In *rar1* and *rar2* plants, this is demonstrated as an intermediate level of susceptibility to powdery mildew (Torp and Jorgensen 1986; Jorgensen 1988, 1996). The *rcr1* and *rcr2* mutations weaken resistance against *Cladosporium fulvum*, but neither mutation allows sporulation of the fungus (Hammond-Kosack *et al.* 1994). Plants carrying the *ndr1* mutation allow extensive growth of several previously avirulent races of *Pseudomonas syringae* pv *tomato*; however, these plants can still produce a visible hypersensitive response to some of these bacterial strains (Century *et al.* 1995). Additionally, *ndr1* plants are only partially susceptible to most isolates of *Peronospora parasitica* (Century *et al.* 1995, 1997).

To identify additional components of *R* gene signal transduction pathways, we screened for mutations that suppressed resistance mediated by the *RPS5* gene of Arabidopsis. *RPS5* confers resistance to *P. s. tomato* carrying *avrPphB* (formerly called *avrPph3*, Simonich and Innes 1995). In contrast to previous screens performed with *avrRpt2* and *avrB* (Kunkel *et al.* 1993; Bisgrove *et al.* 1994), we recovered mutations in three loci other than the targeted *R* gene. Here we describe the effect of these mutations on *RPS5* and on several other *R* genes that confer resistance to various strains of *P. s. tomato* and *P. parasitica*.

MATERIALS AND METHODS

Pseudomonas strains and Peronospora isolates: *P. syringae* strains were cultured as described previously (Innes *et al.* 1993). *P. s. tomato* strains carrying *avrB*, *avrB*::Ω, *avrRpt2*, *avrRps4*, and *avrPphB* have been described (Innes *et al.* 1993; Simonich and Innes 1995; Hinsch and Staskawicz 1996). The *P. 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 were as described previously (Bisgrove *et al.* 1994). Mutagenized seeds (M_2 generation) were obtained from M. Estelle (ethyl methanesulfonate-mutagenized and gamma-irradiated seeds), and Lehle seeds (Round Rock, TX; fast-neutron-mutagenized seeds). In all cases, mutagenesis was performed on seeds (M_1 generation), and the plants were allowed to self-fertilize. Seeds from ~500 M_1 plants were pooled to generate bulked M_2 seed lots. A total of 32 lots were screened to identify the *pbs* mutants. The *pbs1-1* mutation was induced by fast neutrons, the *pbs1-2* and *pbs3* mutants were induced by EMS, and the two *pbs2* mutants were induced by gamma irradiation. It is assumed that the two *pbs2* mutants carry identical mutations because they were isolated from the same seed lot (856 plants were screened from this lot).

Plants were inoculated by dipping whole rosettes in a suspension of $\sim 2 \times 10^8$ colony-forming units of *P. s. tomato* per milliliter as described previously (Innes *et al.* 1993). Genotypes of putative mutants were confirmed as being Col-0 and not contaminating susceptible genotypes through use of several microsatellite and codominant cleaved amplified polymorphic sequences (CAPS) 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 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 5 replications were used per plant genotype/*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 using the dip assay. Seeds were collected from individual selfed F_1 and F_2 plants to generate plants for the next generation. Genetic mapping was performed by polymerase chain reaction using oligonucleotide primers designed to amplify microsatellite sequences (Bell and Ecker 1994) or CAPS (Konieczny and Ausubel 1993). These markers have been used by the Arabidopsis community to establish a well-defined genetic map on a set of recombinant, inbred lines derived from a cross between L*er* and Col-0 (the Lister-Dean recombinant inbred map, http://genome-www.stanford.edu/Arabidopsis/ww/home.html/). Restriction endonucleases (New England Biolabs, Beverly, MA) were used according to the manufacturer's instructions, and DNA isolation from F_2 leaf tissue was performed as described previously (Frye and Innes 1998). Map distances in centimorgans were calculated from recombination frequencies by the Kosambi function (Kosambi 1944).

RESULTS

Isolation of *pbs* mutants: To identify disease-resistance mutants, we inoculated ~16,600 mutagenized Col-0 plants by immersion in a suspension of *P. s. tomato* strain DC3000(*avrPphB*). Mutants were identified by the presence of disease symptoms 4–5 days after inoculation. We have previously reported the isolation of two mutant

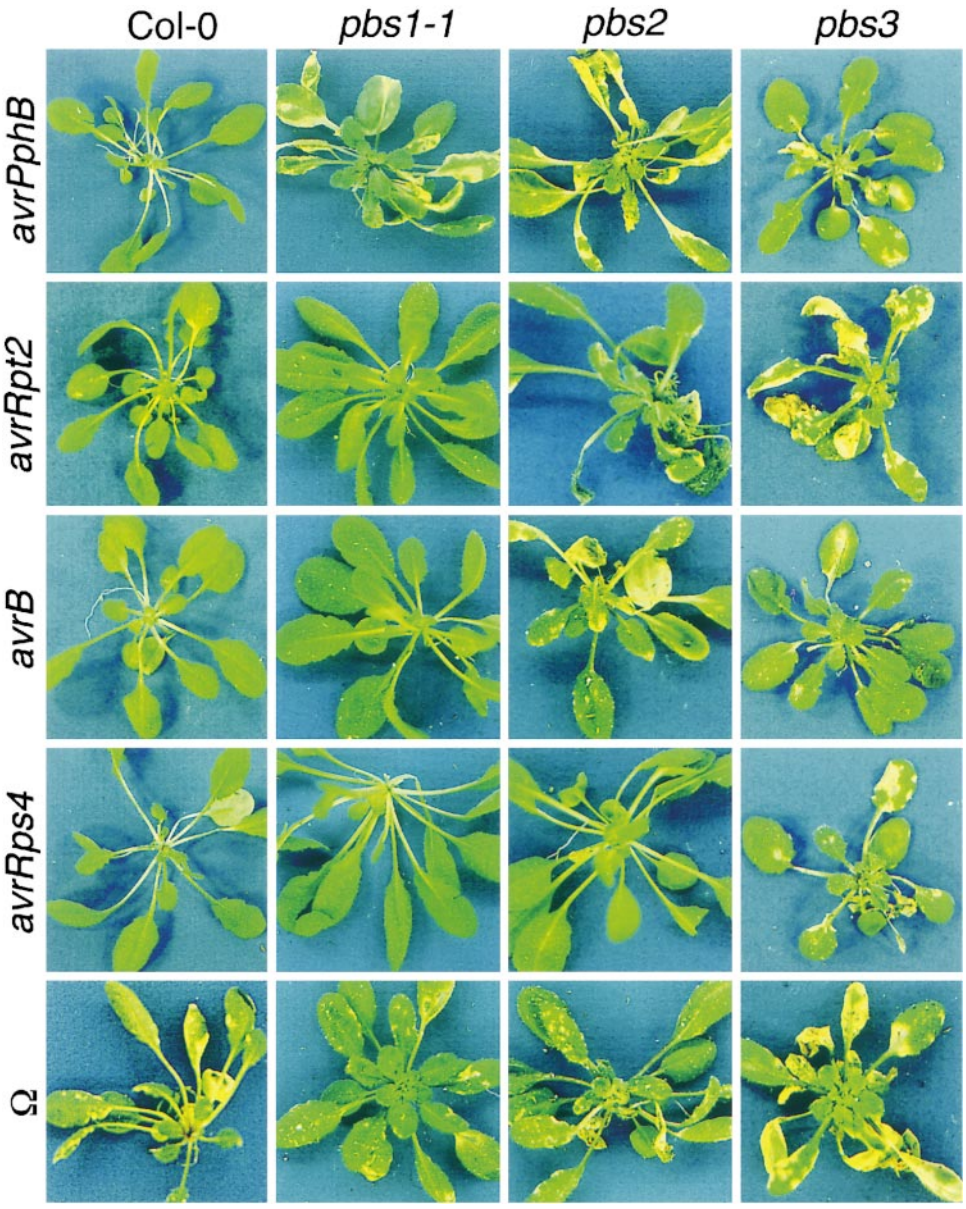

Figure 1.—Disease symptoms induced by *P. s. tomato* strains on *pbs* mutants. The parental accession, Col-0, and the *pbs1-1*, *pbs2*, and *pbs3* mutants were infected by brief submersion in DC3000 strains carrying the indicated avirulence genes. Ω refers to strain DC3000(*avrB*::Ω), which is a virulent control carrying the *avrB* gene that has been disrupted by the insertion of an Ω fragment. Photographs were taken 5 days after inoculation.

plants from this screen that carried mutations within the resistance gene *RPS5* (Warren *et al.* 1998). We also identified five plants that carried mutations in genes other than *RPS5*. As described below, these five mutants represented three complementation groups, which we have designated *pbs1*, *pbs2*, and *pbs3* for *avrPphB* susceptible. Figure 1 shows that *pbs1*, *pbs2*, and *pbs3* plants developed disease symptoms of chlorosis and water-soaked lesions after infection with DC3000(*avrPphB*). Wild-type Col-0 plants remained green and healthy. All self-progeny from the mutants were susceptible to DC3000 (*avrPphB*), indicating that they were homozygous for the mutations.

Genetic analysis of the *pbs* mutants is shown in Table 1. The five mutants were backcrossed to Col-0 plants. All the F_1 plants were resistant to DC3000(*avrPphB*), indicating that the mutations were recessive. The ratio of resistant to susceptible plants in the F_2 generation was determined for each complementation group (see below). Segregation was consistent with a 3:1 ratio for *pbs1*, *pbs2*, and *pbs3* plants, indicating that each susceptible phenotype was caused by a single mutation.

To determine that the mutations were not in *RPS5*, we crossed Col *pbs1*, Col *pbs2*, and Col *pbs3* plants to the Arabidopsis accession Landsberg *erecta* (L*er*), which naturally lacks *RPS5* function (Simonich and Innes 1995). F_1 plants from these crosses were resistant to DC3000(*avrPphB*), and plants in the F_2 generation segregated for resistance. These results demonstrated that the pathogen susceptibility exhibited by mutant plants was not caused by a defect present in the *RPS5* gene.

The *pbs1*, *pbs2*, and *pbs3* complementation groups were established by crossing mutant plants to each other. Mutations were considered allelic if all plants

TABLE 1

Genetic analysis of *pbs* mutants

Cross	F_1		F_2		χ^2
	Resistant	Susceptible	Resistant	Susceptible	
Col-0 × *pbs1-1*[a]	2	0	66	15	1.66 ($P > 0.1$)[b]
Col-0 × *pbs1-2*[a]	18	0	22	10	0.67 ($P > 0.1$)[b]
Col-0 × *pbs2a*[a]	10	0	90	30	0.00 ($P > 0.9$)[b]
Col-0 × *pbs2b*[a]	13	0	—	—	—
Col-0 × *pbs3*[a]	10	0	69	34	3.29 ($P > 0.05$)[b]
Ws-0 × *pbs1-1*[a]	20	0	343	112	0.05 ($P > 0.5$)[b]
Ws-0 × *pbs1-2*[a]	—	—	143	46	0.03 ($P > 0.5$)[b]
Ws-0 × *pbs2b*[a]	—	—	1050	326	1.26 ($P > 0.1$)[b]
L*er* × *pbs1-1*[a]	8	0	103	102	2.85 ($P > 0.05$)[c]
L*er* × *pbs2b*[a]	3	0	75	55	0.12 ($P > 0.5$)[c]
L*er* × *pbs3*[a]	5	0	55	42	0.00 ($P > 0.9$)[c]
L*er* × *pbs3*[d]	—	—	450	198	10.67 ($P < 0.005$)[b]
L*er* × *pbs3*[e]	—	—	132	34	1.80 ($P > 0.1$)[b]
pbs1-1 × *pbs1-2*[a]	0	4	0	154	199.9 ($P < 0.005$)[c]
pbs1-1 × *pbs2a*[a]	2	0	44	50	3.50 ($P > 0.05$)[c]
pbs1-1 × *pbs3*[a]	2	0	13	32	12.96 ($P < 0.005$)[c]
pbs2a × *pbs2b*[a]	0	8	—	—	—
pbs2b × *pbs3*[a]	4	0	5	8	1.24 ($P > 0.1$)[c]

pbs mutants were crossed to the accessions Col-0 and Ws-0, which have *RPS5* function, L*er*, which lacks *RPS5* function, and to each other. Plants were scored as resistant or susceptible on the basis of the presence or absence of water-soaked lesions and chlorosis 4–5 days after inoculation.

[a] Plants were inoculated with *P. s. tomato* strain DC3000(*avrPphB*).

[b] χ^2 values are given for the expected ratio of 3:1.

[c] χ^2 values are given for the expected ratio of 9:7.

[d] Plants were inoculated with *P. s. tomato* strain DC3000(*avrRpt2*).

[e] Plants were inoculated with *P. s. tomato* strain DC3000(*avrB*).

from the resulting generations developed disease symptoms in response to DC3000(*avrPphB*). Of the five mutants isolated, two were placed in the *pbs1* complementation group, two were placed in the *pbs2* complementation group, and one was placed into the *pbs3* complementation group (Table 1). In the case of the two *pbs2* mutants, rather than representing different mutant alleles of the same gene, they likely represent the same mutation since they were isolated from the same pool of mutagenized seed (see materials and methods).

The *pbs* mutants exhibit decreased resistance to multiple *P. s. tomato* strains: In addition to *RPS5*, Col-0 plants possess the *R* genes *RPS2*, *RPM1*, and *RPS4*. These *R* genes 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). To determine if the *pbs* mutations disrupted the function of these other *R* genes, we infected Col-0, Col *pbs1*, Col *pbs2*, and Col *pbs3* plants with DC3000 carrying each of these *avr* genes.

As shown in Figure 1, Col *pbs1-1* plants remained resistant to DC3000 carrying *avrRpt2*, *avrB*, or *avrRps4*. Identical results were obtained with Col *pbs1-2* plants (data not shown). We quantified bacterial growth within Col *pbs1-1* plants, and these data are shown in Figure 2. Consistent with visible symptoms, Col *pbs1-1* plants exhibited enhanced growth only to DC3000(*avrPphB*). These results are similar to those expected for a mutation in *RPS5*, and they suggest that *PBS1* is part of a signal transduction pathway specific to *RPS5*.

In contrast to Col *pbs1* plants, Col *pbs2* plants developed disease symptoms after infection with DC3000 carrying *avrB* or *avrRpt2* (Figure 1). Resistance was not fully compromised to DC3000(*avrRpt2*), which induced less chlorosis and fewer lesions than DC3000(*avrPphB*) or DC3000(*avrB*). Col *pbs2* plants appeared resistant to DC3000(*avrRps4*; Figure 1). In separate trials, these plants were either indistinguishable from wild-type Col-0 plants, or they developed mild disease symptoms that could only be scored on a subset of plants. Thus, for resistance controlled by *RPS4*, the *PBS2* gene product probably does not play a significant role. These results were confirmed by bacterial growth curves (Figure 2). In Col *pbs2* plants, DC3000(*avrPphB*) and DC3000(*avrB*) achieved a level of growth similar to that of a virulent strain of *P. s. tomato*. DC3000(*avrRpt2*) showed slightly elevated growth in Col *pbs2* plants compared to wild-type plants, whereas growth of DC3000(*avrRps4*) was similar in both mutant and wild-type plants.

The increased susceptibility of Col *pbs2* plants to *P. s. tomato* strains carrying *avrPphB*, *avrRpt2*, or *avrB*

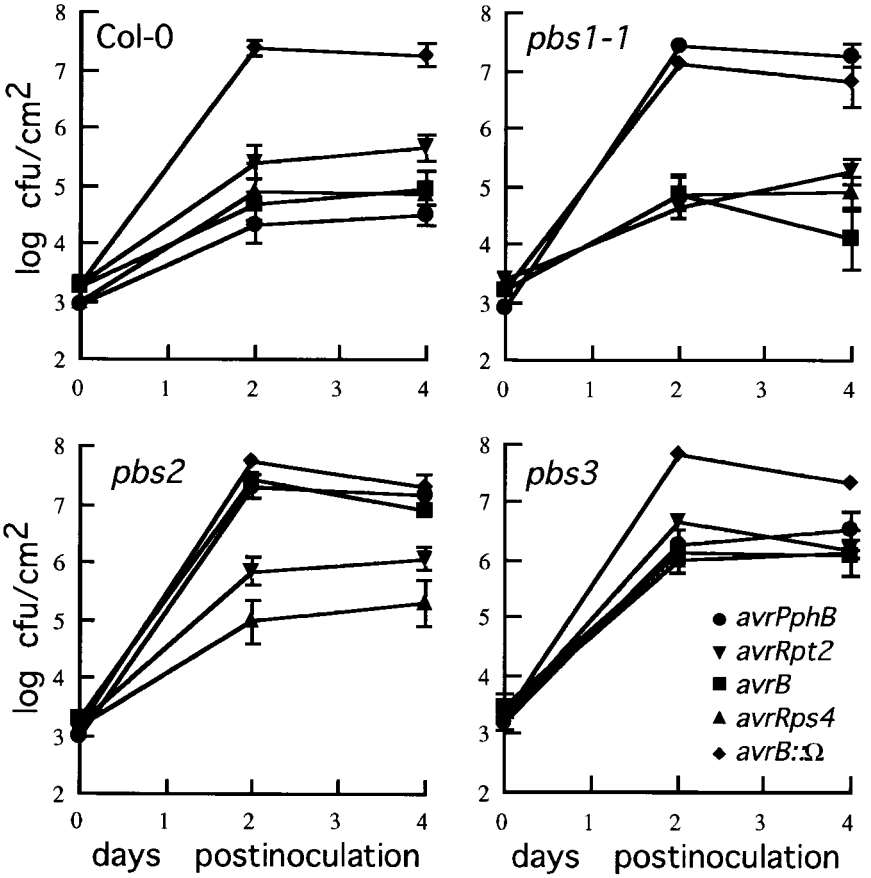

Figure 2.—Growth of *P. s. tomato* strains within leaves of *pbs* mutants. The parental accession, Col-0, and the *pbs1-1*, *pbs2*, and *pbs3* mutants were inoculated by vacuum infiltration, with strain DC3000 carrying the indicated avirulence genes. Growth of bacteria within the leaves was monitored over a 4-day time course. Each data point represents the mean ± SE of three samples. Data shown are representative of two independent experiments.

did not appear to be caused by a second site mutation. We infected F_3 families derived from Col *pbs2* backcrossed plants with DC3000(*avrPphB*), DC3000 (*avrRpt2*), and DC3000(*avrB*). Ten families obtained from DC3000(*avrPphB*)-susceptible F_2 plants developed disease symptoms in response to all three bacterial strains, indicating that the phenotypes were caused by the same or closely linked mutations.

Col *pbs3* plants developed disease symptoms in response to DC3000 carrying *avrRpt2*, *avrB*, or *avrRps4* (Figure 1). DC3000(*avrRpt2*) induced the strongest disease symptoms, and DC3000 carrying *avrPphB*, *avrB*, or *avrRps4* caused less severe disease symptoms. However, resistance was not fully compromised against any of the avirulent pathogens. The bacterial growth of all four avirulent *P. s. tomato* strains was elevated in Col *pbs3* plants, but did not reach the same level of growth as seen for a virulent strain of *P. s. tomato* infecting wild-type Col-0 plants (Figure 2). The decreased resistance to all four *P. s. tomato* strains cosegregated in 15 F_3 families that were derived from either DC3000 (*avrPphB*)- or DC3000(*avrRpt2*)-susceptible F_2 plants.

As shown in Figure 3A, unlike Col *pbs1* and Col *pbs2* plants, Col *pbs3* plants developed more severe disease symptoms than wild-type Col-0 plants when infected by DC3000 containing no added avirulence gene. The growth of this virulent *P. s. tomato* strain in Col *pbs3* leaves is quantified in Figure 3B. Bacterial growth was slightly elevated relative to wild-type Col-0 plants in multiple trials and was statistically significant at 2 days after inoculation. These results suggest that the *PBS3* gene product may be involved in restricting the growth of both virulent and avirulent pathogens.

The *pbs* mutants exhibit decreased resistance to several *P. parasitica* isolates: Because the *pbs2* and *pbs3* mutations affected resistance to multiple *P. s. tomato* strains, we tested whether resistance to the biotrophic oomycete *P. parasitica* (downy mildew) was also affected by the *pbs* mutations. The degree of resistance was measured by counting the number of sporangiophores (tree-like structures emerging from stomata and bearing conidiosporangia) produced in cotyledons. We assessed sporulation in cotyledons of Col *pbs1-2*, Col *pbs2*, and Col *pbs3* seedlings by seven isolates of *P. parasitica*, which are each diagnostic for a different wild-type *RPP* (recognition of *P. parasitica*) gene. As shown in Table 2, differences were observed among the three *pbs* mutants in their response to the seven *P. parasitica* isolates.

Resistance to each of the isolates appeared to be mostly unaffected in Col *pbs1-2* plants. No detectable change from wild type was observed after inoculation with three isolates (Cala2, Hind4, and Hiks1), and a significant but very weak enhanced sporulation was seen with the other four isolates (Emoy2, Wela3, Cand5, and

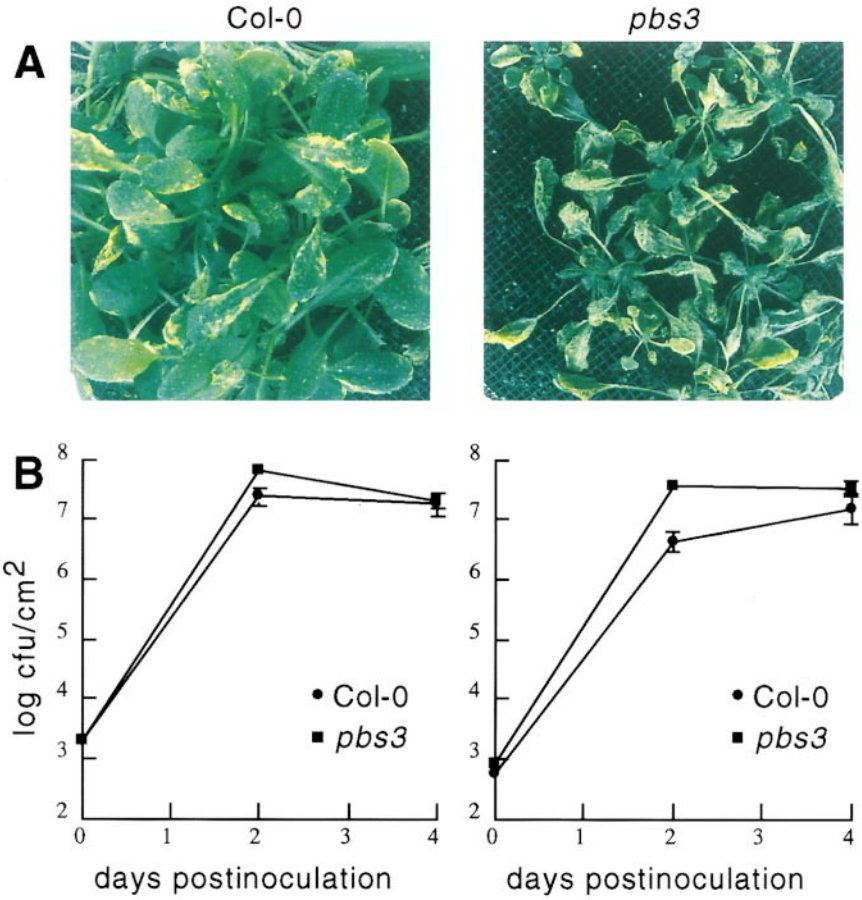

Figure 3.—Response of *pbs3* mutant to virulent *P. s. tomato.* (A) The parental accession, Col-0, and *pbs3* were infected by brief submersion in strain DC3000(*avrB*::Ω), which is a virulent strain of *P. s. tomato* carrying the *avrB* gene that has been disrupted by the insertion of an Ω fragment. Photographs were taken 5 days after inoculation. Col-0 and *pbs3* plants are shown at the same magnification. (B) Growth of DC3000 (*avrB*::Ω) within leaves of Col-0 and *pbs3* plants was monitored over a 4-day time course after inoculation by vacuum infiltration. Each data point represents the mean ± SE of three samples. Data from two independent experiments are shown.

Wand1, Table 2). This enhanced sporulation was much less than that exhibited in a fully susceptible plant, such as Cand5 in Col *ndr1*, which had a mean of at least 20 sporangiophores per cotyledon.

In contrast, the *pbs2* mutation enhanced sporulation to five of the isolates, with an increase to full susceptibility for at least two isolates (Cand5 and Wand1) that produce a rare sporophore or no sporulation, respectively, in wild-type Col-0 cotyledons (Table 2). Medium sporulation was seen with the Emoy2 isolate, and low sporulation was witnessed after inoculation with the Hind4 and Wela3 isolates. Col *pbs2* plants appeared very similar to wild type after inoculation with the remaining two isolates, Cala2 and Hiks1.

Col *pbs3* plants exhibited a third pattern of altered resistance to the isolates (Table 2). Similar to the results obtained with this mutant after bacterial inoculations, resistance to the *P. parasitica* isolates was not fully compromised. However, Emoy2 produced a mean of 16 sporophores per cotyledon compared with a mean of 2 in the wild type, and the mutant was significantly altered to a lesser degree in its response to five other isolates. Cala2 was the only isolate that appeared to exhibit no change in phenotype between the mutant and wild type.

In addition to the *pbs* mutants, we assessed sporulation in Col *ndr1* cotyledons. Similar to the *pbs* mutants, Col *ndr1* plants have been previously reported to exhibit decreased resistance to avirulent *P. s. tomato* strains and downy mildew isolates (Century *et al.* 1995, 1997). Similar to the *pbs* mutants, the level of asexual reproduction varied from no sporulation to heavy sporulation in Col *ndr1* cotyledons, depending on the particular isolate being tested (Table 2). The Col *ndr1* mutants, however, exhibited a pattern of responses to the seven *P. parasitica* isolates that was distinct from that observed in the *pbs* mutants (Table 2).

The *pbs1* and *pbs2* mutations map to chromosomes V and I: Molecular markers were used to determine map positions for the *PBS* genes. The *pbs1-1*, *pbs1-2*, and *pbs2* mutations, present in a Col-0 background, were crossed to the accession Ws-0 that possesses *RPS5* function. F_2 plants homozygous for *pbs1-1*, *pbs1-2*, and *pbs2* mutations were selected on the basis of pathogen susceptibility. As in backcrossed plants, susceptibility to DC3000(*avrPphB*) segregated as a single recessive trait for these mutants (Table 1). DNA was isolated from susceptible plants, and chromosome positions of the *pbs* mutations were established on the basis of linkage to CAPS (Konieczny and Ausubel 1993) and microsatellite (Bell and Ecker 1994) markers. Linkage data are shown in Table 3.

The *pbs1-1* mutation did not map to a discrete location. We identified strong linkage to a region of ~40 cM on both chromosomes IV and V (Table 3). The

TABLE 2

Asexual reproduction in wild-type and mutant lines of Col-0 Arabidopsis by seven *P. parasitica* isolates that are each recognized by a different resistance (*RPP*) gene

Arabidopsis line	*P. parasitica* isolate																							
	Cala2 (*RPP2*-IV)			Emoy2 (*RPP4*-IV)			Hind4 (*RPP19*-II)			Hiks1 (*RPP7*-I)			Wela3 (*RPP6*-I)			Cand5 (not mapped)			Wand1 (not mapped)					
	IP[a]	Mean[b]	SEM	*n*	IP[a]	Mean[b]	SEM	*n*	IP[a]	Mean[b]	SEM	*n*	IP[a]	Mean[b]	SEM	*n*	IP[a]	Mean[b]	SEM	*n*	IP[a]	Mean[b]	SEM	*n*
Col-0	R	0.2	0.0	75	L	1.9	0.2	74	L	2.2	0.3	66	N	0.0	0.0	72	N	0.0	0.0	68	R	0.5	0.1	56	N	0.0	0.0	57
Col *pbs1-2*	R	0.2	0.1	57	L	6.7	0.5	69	L	1.7	0.3	43	N	0.0	0.0	55	R	0.1	0.1	43	L	2.7	0.5	39	R	0.1	0.0	33
Col *pbs2*	R	0.6	0.1	65	M	16.3	0.7	56	L	10.0	0.6	61	N	0.0	0.0	64	L	4.8	0.5	66	H	19.8	0.2	65	H	18.4	0.5	41
Col *pbs3*	R	0.3	0.1	65	M	15.3	0.5	74	L	5.9	0.5	53	R	0.1	0.0	63	R	0.3	0.1	56	L	5.0	0.5	55	L	1.7	0.3	52
Col *ndr1*	R	0.3	0.1	60	M	13.6	0.7	60	L	4.0	0.5	36	R	0.1	0.1	55	L	1.9	0.3	54	H	20.0	0.0	55	L	1.8	0.2	55

RPP loci reviewed in Holub (1997). SEM, standard error of the mean; *n*, number of seedlings inoculated and distributed among five replications.

[a] Interaction phenotype: N, no sporulation; R, rare sporangiophore; L, low sporulation (1–10 sporangiophores per cotyledon); M, medium sporulation (11–18); H, high sporulation (>18).

[b] Mean number of sporangiophores per cotyledon (maximum of 20 counted).

pbs1-1 mutation was induced by fast neutrons, which are known to cause chromosome breaks. The lack of recombination seen on chromosomes IV and V could be explained by a translocation, accompanied by an inversion, between these chromosomes.

In contrast to *pbs1-1*, the *pbs1-2* mutation, which was induced by EMS, mapped to a single region (Table 3). As shown in Figure 4, these data placed *pbs1-2* on chromosome V between the markers nga249 and nga106. The genetic distances between markers was consistent with that derived from the Lister-Dean recombinant inbred lines, indicating no suppression of recombination in *pbs1-2*.

We determined that the *pbs2* mutation was located on chromosome I. On the basis of recombination breakpoints, *pbs2* was placed between the markers nga63 and NCC1 in a genetic interval of <0.4 cM (Table 3). *RPS5*, which confers resistance to DC3000(*avrPphB*), is also located near this region (Figure 4, Simonich and Innes 1995).

The *pbs3* mutation exhibits partial dominance and maps to chromosome V: When Col *pbs3* plants were crossed to the Arabidopsis accessions Col-0, Col *pbs1-1*, Col *pbs2*, and L*er*, all plants in the resulting F_1 generation appeared resistant to DC3000(*avrPphB*), indicating that *pbs3* was recessive. However, segregation of the mutant trait in the F_2 generation of some of these crosses deviated significantly from expectations (Table 1). Susceptible plants were predominant in the F_2 generation resulting from the cross of Col *pbs3* to Col *pbs1-1*. Assuming the *pbs3* and *pbs1-1* mutations are unlinked and recessive, the expected ratio of resistant to susceptible plants would be 9:7. We identified 13 resistant plants and 32 susceptible plants ($\chi^2 = 12.96$), which is not statistically consistent with a 9:7 ratio. In this cross, the skewed segregation could result from a genetic interaction between the *pbs1-1* and *pbs3* alleles. Additionally, because *pbs1* is linked to *pbs3* (see below), the inversion/translocation that may be present in the *pbs1-1* background could affect the segregation of *pbs3* in this cross. Segregation of resistance in the backcross to Col-0 was consistent with a 3:1 ratio (69 resistant:34 susceptible; $\chi^2 = 3.29$), but the actual number of resistant to susceptible plants was closer to a 2:1 ratio. Segregation did not deviate significantly from 9:7 in the cross to Col *pbs2*, but only a few plants were assayed in the F_2 generation (Table 1). Taken together, we interpret these data to indicate that plants heterozygous for the *pbs3* mutation may have slightly enhanced susceptibility that sometimes causes a susceptible phenotype, depending on genetic and/or environmental variables.

To map the *pbs3* mutation, we used a cross to the Arabidopsis accession L*er*. Because L*er* lacks *RPS5*, we scored for the *pbs3* mutant phenotype using DC3000 strains containing *avrRpt2* or *avrB* rather than *avrPphB*. For DC3000(*avrRpt2*), segregation of resistant to susceptible plants was not consistent with a 3:1 ratio. Four

TABLE 3

Frequency of recombination between *pbs* mutations and molecular markers

pbs mutation	Recombination events	Total number of meioses counted	Recombination frequency (%)
pbs1-1[a]			
nga8-IV(24)	1	108	9.3
g4539-IV(55)	0	108	0.0
g3883-IV(61)	1	104	0.9
nga1107-IV(102)	38	110	34.5
nga158-V(15)	4	110	3.6
nga249-V(23)	0	110	0.0
nga151-V(29)	0	110	0.0
nga106-V(33)	0	110	0.0
nga139-V(55)	2	106	1.9
nga129-V(107)	39	110	35.5
pbs1-2[a]			
nga8-IV(24)	18	44	40.9
g4539-IV(55)	13	32	40.6
nga249-V(23)	3[b]	46	6.5
nga151-V(29)	3[b]	56	5.4
nga106-V(33)	3	68	4.4
nga139-V(55)	14	38	36.8
pbs2[a]			
ateat1-I(2)	6	152	3.9
nga63-I(9)	1	536	0.2
ncc1-I(10)	1	506	0.2
pbs3[a]			
nga225-V(12)	7	172	4.1
asa1-V(15)	4	122	3.2
nga249-V(23)	1	158	0.6
nga151-V(29)	7	172	4.1
nga106-V(33)	9	124	7.3

[a] For each *pbs* mutant, the CAPS or microsatellite marker is listed, followed by the chromosome number where each particular marker is located. Shown in parentheses is the map position in centimorgans assigned to the marker in the Lister-Dean recombinant inbred map.

[b] The three recombinantion events listed for *pbs1-2* for the nga249 and nga151 markers are mutually exclusive.

hundred fifty plants were scored as resistant, and 198 plants were scored as susceptible ($\chi^2 = 10.67$). Eighty-one DC3000(*avrRpt2*)-susceptible plants were tested initially for linkage, and the results suggested that *pbs3* was located on chromosome V, near the marker nga249. Plants showing recombination near this region were retested for their response to DC3000(*avrRpt2*) in the F_3 generation. Fifteen plants (19%) segregated for resistance, indicating they were heterozygous for *pbs3*, and they were not included in the linkage data shown in Table 3.

An identical analysis was performed on DC3000 (*avrB*)-susceptible plants. Segregation was consistent with a single recessive gene (132 resistant:34 susceptible; $\chi^2 = 1.80$), but some susceptible plants were probably not identified because symptom development in response to DC3000(*avrB*) is weaker than that seen with DC3000(*avrRpt2*) (Figure 1). Because DC3000(*avrRpt2*) induces a stronger phenotype, it was used to infect recombinant plants in the F_3 generation. Of the 28 susceptible plants analyzed for linkage, 6 (21%) segregated for resistance and were not included in the data shown in Table 3.

After the elimination of plants that segregated for disease resistance in the F_3 generation, *pbs3* was placed to a single genetic locus on chromosome V near the same region as *pbs1-2* (Figure 4), between the markers nga249 and nga151. Recombination frequencies indicated *pbs3* was ~0.6 cM from nga249 and 4.1 cM from nga151 (Table 3).

DISCUSSION

We have used a mutational approach to characterize molecular pathways leading to disease resistance in Arabidopsis. Three new genes were identified that exhib-

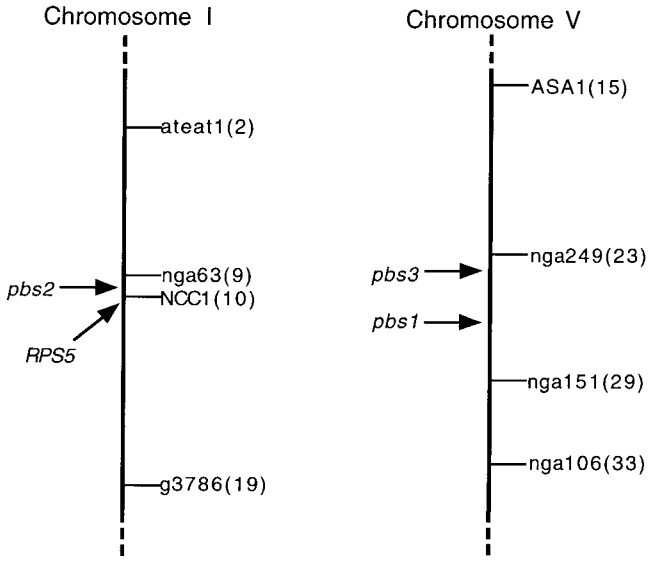

Figure 4.—Chromosome positions of the *pbs1*, *pbs2*, and *pbs3* mutations. The map location of the *pbs* mutations relative to CAPS and microsatellite markers is shown. In parentheses is the position of each marker in centimorgans according to the Lister-Dean recombinant inbred map.

ited susceptibility to several previously avirulent pathogens. The *pbs1* mutation conferred full susceptibility to only one avirulent pathogen (Figures 1 and 2, Table 2), indicating that this gene product may be critical to only one *R* gene-induced resistance pathway. The *pbs2* and *pbs3* mutant plants were susceptible to varying degrees against races of both prokaryotic and eukaryotic pathogens (Figures 1 and 2, Table 2), suggesting that these two genes fulfill a function common to several *R* gene pathways.

PBS2 is genetically linked to *RPS5*, and *PBS1* is linked to *PBS3*. The presence of functionally associated genes near the same genetic location has been observed before. For example, the *R* gene *Pto* is genetically linked to the *Prf* gene, which is required for *Pto* function (Salmeron *et al.* 1994). Similarly, two genes involved with self-incompatibility in Brassica are also tightly linked (Boyes and Nasrallah 1993). For such examples, an argument can be made that the linked genes are both highly variable and specific to each other and, thus, must be inherited together to be functional. The *PBS* genes do not conform to this logic, however, as *PBS2* and *PBS3* mutations affect unlinked *R* genes.

PBS1 is closely associated with RPS5-mediated pathogen recognition: Pathogen recognition mediated by *R* genes of the nucleotide-binding site (NBS)/leucine-rich repeat (LRR) class may require specific kinase partners (Innes 1995). To date, the involvement of both of these components has been shown only in the tomato for resistance mediated by the *R* gene *Pto*. *Pto* confers resistance against *P. s. tomato* carrying *avrPto* and encodes a functional serine/threonine kinase (Ronald *et al.* 1992; Loh and Martin 1995). Consistent with a postulated role as a receptor, Pto interacts with AvrPto in the yeast two-hybrid system (Scofield *et al.* 1996; Tang *et al.* 1996).

In addition to Pto, recognition of AvrPto requires *Prf*, which encodes an NBS/LRR protein (Salmeron *et al.* 1996). *Prf* was originally identified in a screen for tomato mutants susceptible to *P. s. tomato* carrying *avrPto* (Salmeron *et al.* 1994). Mutations within *Prf* can fully suppress resistance mediated by *Pto*. An interaction between Pto and Prf has not been shown, but it has been suggested that the coupling of kinase and NBS/LRR components could confer specificity to a particular receptor complex (Innes 1995; Salmeron *et al.* 1996).

Similar to *Prf* and *Pto*, we have now identified two genes required for the recognition of *avrPphB*, *RPS5*, and *PBS1*. Since, like many *R* genes, *RPS5* encodes an NBS/LRR protein (Warren *et al.* 1998), a kinase is a candidate to be encoded by *PBS1*. Regardless of its structure, PBS1 is likely to be closely associated with the recognition of an *avrPphB*-derived elicitor because the *pbs1* mutations fully suppressed disease resistance conferred by *RPS5*. Also, unlike most putative *R* gene signal transduction mutations that have been isolated, including *pbs2* and *pbs3*, the *pbs1* mutations did not greatly affect *R* genes other than *RPS5* (Figures 1 and 2, Table 2). A slight increase in sporulation was observed after inoculation with the *P. parasitica* isolates Emoy2 and Cand5 (Table 2), which may indicate that the *PBS1* gene product can exhibit some specificity toward these *RPP* gene products.

PBS2 and NDR1 are involved in the same signal transduction pathways: Like *pbs2*, the Arabidopsis mutation *ndr1* affects *R* genes that specify resistance to avirulent *P. s. tomato* and *P. parasitica*. The *pbs2* mutation appears to suppress the same set of *R* genes as *ndr1*. Both *pbs2* and *ndr1* exhibit increased disease symptoms in response to *P. s. tomato* carrying *avrPphB*, *avrRpt2*, and *avrB* (Figure 1; Century *et al.* 1995). Additionally, neither mutation allows increased growth of *P. s. tomato* carrying *avrRps4* (Figure 2; Aarts *et al.* 1998). Both *pbs2* and *ndr1* plants also show increased susceptibility to the same *P. parasitica* isolates (Table 2). For example, both mutants allow medium-to-high sporulation of Emoy2 and Cand5 on cotyledons and low sporulation of Hind4 and Wela3. The *ndr1* mutation is not allelic to any of the *pbs* mutations, however, because they map to different chromosome locations (Table 3; Century *et al.* 1995).

Given that the same *R* genes are affected by *pbs2* and *ndr1*, these gene products may be closely associated with each other in the same signal transduction pathways. The precise role of *NDR1* in pathogen resistance is currently unknown, as the *NDR1* protein does not exhibit similarity to proteins of known function (Century *et al.* 1997). However, *NDR1* mRNA accumulation increases after infection by virulent or avirulent *P. s. tomato* strains and probably functions downstream of initial pathogen recognition.

Although the *ndr1* and *pbs2* mutations appear to affect the function of the same set of *R* genes, these gene products are not identical in their importance to all *R* gene signal transduction pathways. For example, *NDR1* appears to be more critical than *PBS2* for resistance specified by *RPS2*. Growth of *P. s. tomato* strain DC3000(*avrRpt2*) appears to be unrestricted in *ndr1* leaves (Century *et al.* 1995), but *pbs2* plants were not fully susceptible to this pathogen (Figure 2). The *pbs2* mutation also suppresses resistance against the *P. parasitica* isolate Wand1 much more strongly than does *ndr1* (Table 2).

PBS3 acts to restrict growth of virulent and avirulent pathogens: Col *pbs3* mutant plants exhibited more severe disease symptoms in response to a virulent strain of *P. s. tomato* than did wild-type Col-0 plants (Figure 3). This enhanced disease susceptibility suggests that *PBS3* is involved in controlling the growth of both virulent and avirulent pathogens.

Several Arabidopsis mutants have been isolated that show enhanced susceptibility to a virulent pathogen, and some of these mutations also affect resistance to avirulent pathogens (Cao *et al.* 1994; Glazebrook and Ausubel 1994; Delaney *et al.* 1995; Glazebrook *et al.* 1996, 1997). For example, *eds1* mutants exhibit enhanced susceptibility to virulent *P. parasitica* and *P. s. tomato* (Parker *et al.* 1996; Aarts *et al.* 1998). Additionally, *eds1* mutations have been shown to disrupt resistance mediated by eight different *RPP* loci and a single bacterial resistance locus, *RPS4*. In contrast to *eds1*, the *pbs3* mutation partially suppressed resistance conferred by four bacterial resistance genes, including *RPS4*, and allowed medium levels of sporulation on cotyledons by only one of the *P. parasitica* isolates that was tested and low sporulation by three other isolates (Figures 1 and 2, Table 2). The Arabidopsis mutants *pad1*, *pad2*, *pad4*, and *npr1* all show enhanced susceptibility to virulent *P. s. maculicola* (Cao *et al.* 1994; Glazebrook and Ausubel 1994; Glazebrook *et al.* 1996, 1997). None of these mutants are altered in their response to avirulent bacteria, but their resistance is affected to different degrees against avirulent isolates of *P. parasitica* (Delaney *et al.* 1995; Glazebrook *et al.* 1997). These differences in phenotype suggest that *PBS3* encodes a signaling component that is distinct from that encoded by *EDS1*, *NPR1*, or the *PAD* genes.

The phenotypes seen in Col *pbs3* plants are reminiscent of plants with reduced levels of salicylic acid (SA). Transgenic Col-0 plants producing salicylate hydroxylase, which degrades SA, show enhanced susceptibility to virulent and avirulent *P. s. tomato* and *P. parasitica* pathogens (Delaney *et al.* 1994). However, *pbs3* does not seem to completely abolish SA-dependent defense responses because *pbs3* plants are only partially suppressed in resistance to DC3000(*avrRpt2*) (Figure 2), while Col-0 plants expressing salicylate hydroxylase allow growth of DC3000(*avrRpt2*) equivalent to that seen in susceptible accessions of Arabidopsis (Delaney *et al.* 1994). It is plausible that *pbs3* plants have intermediate levels of SA.

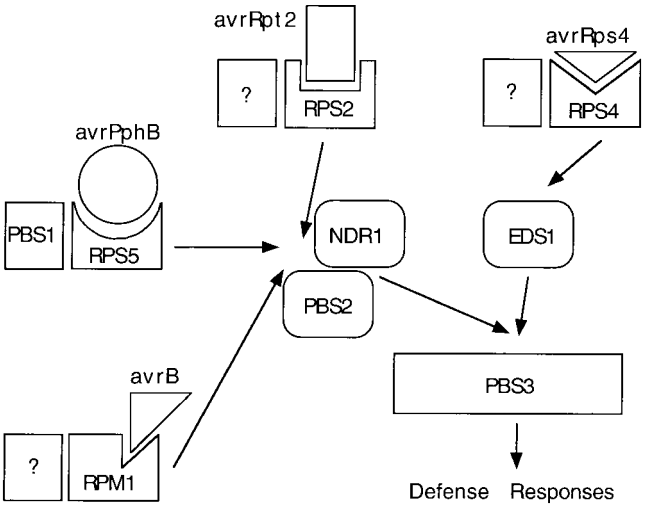

Figure 5.—Summary model of *R* gene disease-resistance pathways in Arabidopsis. The proposed placement of gene products in this pathway is based on phenotype analysis. These pathways are activated by an *avr* gene product derived from *P. syringae*. The recognition of this *avr*-based signal by RPS5 requires the *PBS1* gene product. Similar proteins may be required by RPS2, RPM1, and RPS4, but they have not been identified. Potential roles of PBS2, PBS3, NDR1, and EDS1 in *RPP* gene-mediated resistance pathways are not shown.

***PBS* gene-dependent signal transduction pathways:** The identification of three new genes required by Arabidopsis to induce disease resistance allowed the dissection of signal transduction pathways that are activated by avirulent pathogens. A summary model based on assessment of *R* gene function disrupted by the *pbs* mutations and comparison to *ndr1* and *eds1* mutants is presented in Figure 5. In this model, the *PBS1* gene product is closely associated with recognition of an *avrPphB*-derived elicitor, while the *PBS2* and *PBS3* gene products function downstream of pathogen recognition in multiple *R* gene defense pathways.

The isolation of *PBS1*, which completely abolishes the function of a single *R* gene, *RPS5*, suggests that analogous genes could be identified by conducting mutant screens that assayed for loss of resistance conferred by other *R* genes. In Arabidopsis, however, genetic screens for loss of *RPS2*-, *RPM1*-, or *RPP5*-mediated disease resistance did not identify a mutation similar to *pbs1* (Kunkel *et al.* 1993; Yu *et al.* 1993; Bisgrove *et al.* 1994; Century *et al.* 1995; Parker *et al.* 1996). This may suggest that PBS1 performs a function unique to the RPS5 disease-resistance pathway, or that redundant gene products perform its role in other *R* gene signal transduction pathways.

The simplest interpretation of the effects of the *pbs2* and *pbs3* mutation suggests that *PBS3* operates downstream of *PBS2* (Figure 5). It is also possible, however,

that *PBS3* is involved in an independent pathway that contributes to resistance. The enhanced susceptibility to virulent pathogens and the failure of the *pbs3* mutation to completely abolish disease resistance supports this idea. Continued analysis of the *pbs* mutants and characterization of the corresponding gene products should further our understanding of the processes used by plants to limit pathogen growth.

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