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Use of *Arabidopsis thaliana* defense-related mutants to dissect the plant response to pathogens

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ABSTRACT **The plant defense response to microbial pathogens had been studied primarily by using biochemical and physiological techniques. Recently, several laboratories have developed a variety of pathosystems utilizing *Arabidopsis thaliana* as a model host so that genetic analysis could also be used to study plant defense responses. Utilizing a pathosystem that involves the infection of *Arabidopsis* with pathogenic pseudomonads, we have cloned the *Arabidopsis* disease-resistance gene *RPS2*, which corresponds to the avirulence gene *avrRpt2* in a gene-for-gene relationship. *RPS2* encodes a 105-kDa protein containing a leucine zipper, a nucleotide binding site, and 14 imperfect leucine-rich repeats. The RPS2 protein is remarkably similar to the product of the tobacco *N* gene, which confers resistance to tobacco mosaic virus. We have also isolated a series of *Arabidopsis* mutants that synthesize decreased levels of an *Arabidopsis* phytoalexin called camalexin. Analysis of these mutants indicated that camalexin does not play a significant role in limiting growth of avirulent *Pseudomonas syringae* strains during the hypersensitive defense response but that it may play a role in limiting the growth of virulent strains. More generally, we have shown that we can utilize *Arabidopsis* to systematically dissect the defense response by isolation and characterization of appropriate defense-related mutants.**

Plants respond in a variety of ways to pathogenic microorganisms (1). Infected cells undergo rapid programmed cell death called the hypersensitive response (HR). Cell walls are reinforced by lignification, suberization, callose deposition, and cross-linking of hydroxyproline-rich proteins. A variety of hydrolytic enzymes and low molecular weight antibiotics (called phytoalexins) are synthesized. A membrane-associated oxidative burst occurs that results in the NADPH-dependent production of O_2^- and H_2O_2. Moreover, the activation of particular plant genes accompanies each of these responses. Although a large body of work has shown that these responses are often correlated with disease resistance, it has been difficult to provide compelling evidence for the significance of particular responses in conferring resistance. Moreover, although the structures of several pathogen-derived elicitors of the plant defense response have been determined, the components of the signal transduction pathways that lead to the activation of defense responses are only beginning to be identified.

One aspect of the plant defense response where genetic analysis has played an important role is in the breeding of pathogen resistance in economically important plants. In many plant–pathogen interactions, the visible hallmark of pathogen resistance is activation of the HR. Pathogens that elicit a HR on a given host are said to be avirulent on that host, the host is said to be resistant, and the plant–pathogen interaction is said to be incompatible. In contrast, strains that proliferate and cause disease on a particular host are said to be virulent; in this case, the host is said to be susceptible, and the plant–pathogen interaction is said to be compatible. In many cases in which a series of strains (races) of a particular pathogen are either virulent or avirulent on a series of cultivars (or different wild accessions) of a particular host species, genetic analysis has revealed a gene-for-gene correspondence between pathogen avirulence (*avr*) genes and host "resistance" genes (2). In other words, a plant carrying a particular resistance gene will be resistant to pathogens carrying the corresponding *avr* gene. A simple explanation for this gene-for-gene correspondence is that *avr* genes generate signals for which resistance genes encode the cognate receptors. A signal transduction pathway then carries the *avr*-generated signal to a set of target genes, which initiates the HR and other host defenses (1, 3, 4).

A variety of *avr* genes have been cloned from bacterial and fungal phytopathogens (4) and, in at least two cases, gene-for-gene interactions have been demonstrated at the molecular level by demonstrating that a purified *avr*-generated signal molecule will elicit a HR. In the best documented case, two *avr* genes from the tomato fungal pathogen *Cladosporium fulvum*, *avr9* and *avr4*, were shown to encode precursors of elicitor peptides. Purified avr9 and avr4 peptides specifically elicit a HR in tomato plants that harbor the corresponding resistance genes, *Cf9* and *Cf4*, respectively (5, 6). The only example of a bacterial *avr* gene where the *avr*-generated signal has been identified is the *Pseudomonas syringae* pv. *glycinea avrD* locus, which encodes biosynthetic enzymes involved in the synthesis of specific syringolides that elicit a HR in soybean cultivars carrying the resistance gene *Rpg4* (4, 7). In the case of tobacco mosaic virus, the viral-encoded coat protein appears to function as a specific elicitor that activates a HR in *Nicotiana sylvestris* cultivars that carry the N′ resistance gene (8, 9).

Until recently, the only cloned resistance gene that is demonstrably involved in a gene-for-gene relationship was the tomato *PTO* gene (10). The *PTO*-encoded protein, which corresponds to the *P. syringae avrPto* gene, consists almost entirely of a serine/threonine kinase domain. Although the structure of PTO protein suggests involvement of protein phosphorylation in the signal transduction pathway leading to resistance responses, it is not known whether this PTO kinase also serves as the primary receptor for an *avrPto*-generated signal. As discussed below, additional resistance genes have recently been cloned from *Arabidopsis* and tobacco.

Because plant–pathogen interactions necessarily involve two organisms, it would be desirable to be able to genetically manipu-

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Abbreviations: HR, hypersensitive response; cfu, colony-forming units; LRR, leucine-rich repeat.

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late both the pathogen and its host to facilitate the dissection of various aspects of plant–pathogen interactions. To accomplish this goal, several laboratories have recently turned to the easily manipulated crucifer *Arabidopsis thaliana* (11) as a model to study the plant response to pathogen attack. *Arabidopsis* offers several advantages compared to other plants that have been used previously to study plant–pathogen interactions. The small stature, fast generation time, copious production of tiny (20 μg) seeds, and the small (100 Mb) genome of *Arabidopsis* facilitate the use of genetic strategies to identify defense-related mutants and the use of gene tagging or map-based positional cloning strategies to isolate the corresponding genes.

Significant progress has been made in establishing *Arabidopsis* pathogenesis models for a variety of bacterial, fungal, and viral pathogens (12–29). In addition, an *Arabidopsis* phytoalexin has been identified that is toxic to both bacteria and fungi (27) and a variety of *Arabidopsis* pathogen-induced genes have been identified for use in monitoring the *Arabidopsis* defense response. *Arabidopsis* pathogen-induced genes include ones corresponding to tobacco pathogenesis-related (PR) proteins and to parsley elicitor-induced (ELI) cDNAs (13, 30–36), *PAL1* [phenylalanine ammonia lyase (14, 37)], *BGL2* [β-1,3-glucanase (16)], *SOD1* [superoxide dismutase (38)], *LOX1* [lipoxygenase (39)], *DHS1* (3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (40)], and *ASA1* [anthranilate synthase (41)].

In this article, we summarize how our laboratory has used a model system that involves the infection of *Arabidopsis* with phytopathogenic pseudomonads to isolate two categories of *Arabidopsis* defense-related mutants, which illuminate two features of the plant defense response. First, we have identified an *Arabidopsis* disease resistance locus by isolating *Arabidopsis* mutants that failed to mount a HR when infected with *P. syringae* expressing a particular avirulence gene (42). In this case, we have cloned the identified resistance gene by using a map-based chromosomal walking strategy (43). Second, we have identified *Arabidopsis* genes involved in the biosynthesis of a phytoalexin by screening for *Arabidopsis* mutants that synthesize reduced levels of the phytoalexin (44). In this case, characterization of the mutant phenotypes suggests a role for phytoalexins in limiting the growth of *P. syringae* during a compatible interaction.

The *Arabidopsis–P. syringae* Model

Several laboratories, including ours, have pioneered the use of *Arabidopsis* for the study of the plant response to phytopathogenic *Pseudomonas* species (14–16, 29). We have shown that the virulent bacterial pathogen *P. syringae* pv. *maculicola* (*Psm*) ES4326 proliferates extensively in *Arabidopsis* leaves and causes the development of disease symptoms (14, 16). In contrast, *Psm* ES4326 expressing the *avr* gene *avrRpt2*, cloned from a different *P. syringae* strain (16, 29), elicits a HR and grows only ≈1% as much in *Arabidopsis* leaves as *Psm* ES4326 not expressing *avrRpt2*. The *Arabidopsis* resistance gene *RPS2* corresponding to *avrRpt2* was identified by screening for *Arabidopsis* mutants that failed to mount a resistance response when infected with *Psm* ES4326/*avrRpt2* (42, 45). Naturally occurring *rps2* mutant alleles in various *Arabidopsis* ecotypes have also been identified (45, 46).

Fig. 1 *A–F* illustrates the symptomology observed on susceptible and resistant *Arabidopsis* plants when infiltrated with virulent and avirulent *P. syringae* strains. In contrast to wild-type (Fig. 1*A*), *rps2* mutants fail to mount a HR 16 hr after infiltration with *Psm* ES4326/*avrRpt2* (Fig. 1*B*) but still mount a HR when infiltrated with *Psm* ES4326/*avrRpm1* (Fig. 1*C*). The *avrRpm1* gene is an independently identified *avr* gene that generates a signal to which *Arabidopsis* responds with a HR (15). Although *rps2* mutants exhibit no symptoms 16 hr after infiltration with *Psm* ES4326/*avrRpt2*, disease symptoms become visible after 48 hr (Fig. 1*D*) that are indistinguishable from those caused by infiltration of wild-type leaves with *Psm* ES4326 after 48 hr (Fig. 1*E*). These types of results suggest that *rps2* mutants do not have general defects in the ability to mount a HR but rather specifically fail to recognize an *avrRpt2*-generated signal, causing them to be susceptible to *Psm* ES4326/*avrRpt2*.

Because the elicitation of visible HR symptoms by an avirulent strain depends on the inoculation dose, we have used Evan's blue dye, which is excluded from intact cells, to monitor the death of individual plant cells after infiltration of leaves with doses of *P. syringae* strains that are too low to elicit a visible HR. Fig. 1 *F–H* shows Evan's blue staining in wild-type leaves after infiltration with *Psm* ES4326/*avrRpt2* at three different doses. At a very low infiltration dose (50 cells per cm^2 leaf area) widely spaced individual stained cells are observed (Fig. 1*H*). At a 40-fold higher infiltration titer [2×10^3 colony-forming units (cfu)/cm^2], which is still too low to elicit a visible HR, a mosaic pattern of stained and unstained cells is observed (Fig. 1*G*). Fig. 1*F* shows the boundary between infiltrated and noninfiltrated tissue in a leaf that developed a visible HR 16 hr after infiltration at a dose of 2×10^4 cfu/cm^2. The totally collapsed tissue that was infiltrated at the top of Fig. 1*F*, did not stain, but the cells that comprised the boundary between the infected and the uninfected portion of the leaf stained blue. In contrast to wild type, Fig. 1*I* shows that no Evan's blue staining is observed in *rps2* mutant leaves infiltrated with *Psm* ES4326/*avrRpt2* at 2×10^3 cfu/cm^2, the same titer that elicits extensive staining in wild-type leaves (Fig. 1*G*). Similar results are obtained with infiltration doses as high as 2×10^4 cfu/cm^2 (data not shown). Leaves infiltrated with *Psm* ES4326 did not stain within 16 hr after infiltration; staining of individual cells was first apparent after 30 hr and extensive staining occurred by 48 hr concomitantly with the appearance of visible disease symptoms (data not shown).

One method used to isolate *rps2* mutants was notable for the use of a strategy that took advantage of the small size of *Arabidopsis* seeds (42). This strategy for isolating *rps2* mutants was based on the observation that the extensively studied bean pathogen *P. syringae* pv. *phaseolicola* (*Psp*) strain NPS3121, although not an *Arabidopsis* pathogen, elicits a strong HR when expressing *avrRpt2*. When 10-day-old *Arabidopsis* seedlings growing on Petri plates are infiltrated with *Psp* NPS3121 and *Psp* NPS3121/*avrRpt2*, ≈90% of the plants infiltrated with *Psp* NPS3121 survive, whereas 90–95% of the plants infiltrated with *Psp* NPS3121/*avrRpt2* die. Apparently, vacuum infiltration of an entire small *Arabidopsis* seedling with *Psp* NPS3121/*avrRpt2* elicits a systemic HR, which usually kills the seedling. In contrast, seedlings infiltrated with *Psp* NPS3121 survive because *Psp* NPS3121 is such a weak pathogen on *Arabidopsis*. In actual practice, when ≈4000 ethyl methanesulfonate-mutagenized Columbia M2 seedlings (≈200 seedlings per plate) were infiltrated with *Psp* NPS3121/*avrRpt2*, ≈200 survivors were obtained. When these survivors were transplanted to soil and rescreened by hand inoculation with *Psm* ES4326/*avrRpt2* when the plants reached maturity, one plant failed to mount a HR (42). Subsequent genetic analysis showed that this plant, *rps2-102C*, carried a mutation at the *RPS2* locus (42).

An interesting phenotype of all of the *rps2* mutant alleles studied to date is that they are incompletely recessive with respect to wild-type alleles (42, 45, 46). For example, *RPS2/rps2* heterozygotes mount a HR in response to *Psm* ES4326/*avrRpt2*; however, a higher inoculum is required to elicit the HR compared to wild type (*RPS2/RPS2*) and visible HR symptoms appeared later (24–30 hr) in heterozygous plants than in *RPS2/RPS2* plants (14–18 hr). Moreover, when heterozygous *RPS2/rps2* plants are infiltrated with a relatively low dose of *Psm* ES4326/*avrRpt2* (2×10^3 cfu/cm^2), disease symptoms appear after 48 hr, whereas *RPS2/RPS2*

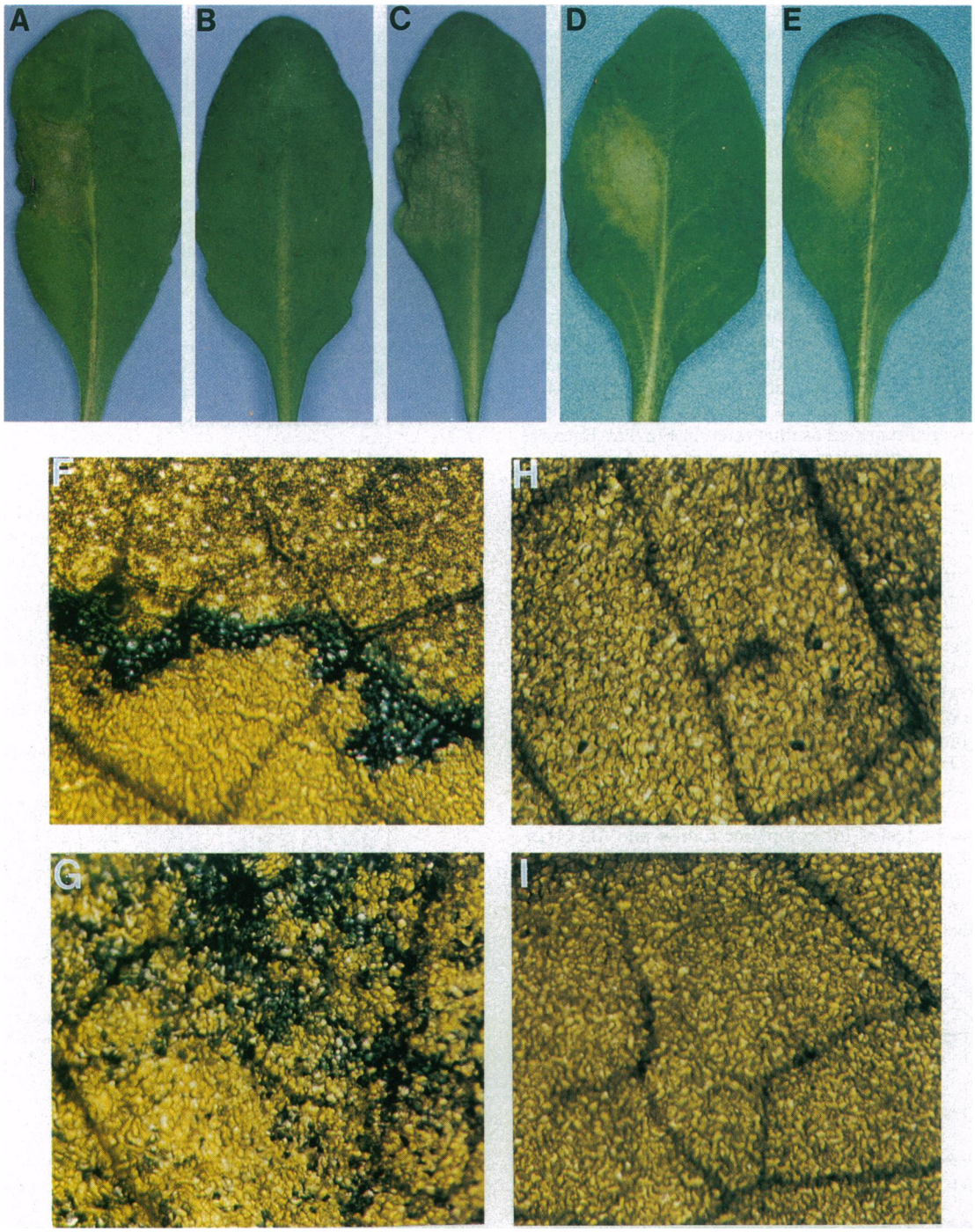


FIG. 1. Macroscopic and microscopic symptoms elicited by *P. syringae* strains infiltrated into *Arabidopsis* leaves. (*A–E*) *Arabidopsis* leaves were infiltrated with bacterial suspensions at a titer of 2×10^4 cfu/cm^2 and photographed 16 or 48 hr postinfiltration. (*A*) Wild-type leaves infiltrated with *Psm* ES4326/*avrRpt2* at 16 hr. (*B*) *rps2* mutant leaves infiltrated with *Psm* ES4326/*avrRpt2* at 16 hr. (*C*) *rps2* mutant leaves infiltrated with *Psm* ES4326/*avrRpm1* at 16 hr. (*D*) *rps2* mutant leaves infiltrated with *Psm* ES4326/*avrRpt2* at 48 hr. (*E*) Wild-type leaves infiltrated with *Psm* ES4326 at 48 hr. (*F–I*) *Arabidopsis* leaves were infiltrated with bacterial suspensions at various titers and stained with Evan's blue after 16 hr. Whole leaves previously hand infiltrated with a bacterial suspension were vacuum infiltrated with 0.25% Evan's blue dye, rinsed three or four times with water to remove excess dye, left overnight at room temperature in water, and examined and photographed with a dissecting microscope. (×110.) (*F–H*) Wild-type leaves infiltrated with *Psm* ES4326/*avrRpt2* at 2×10^4, 2×10^3, and 0.5×10^2 cfu/cm^2, respectively. (*I*) *rps2* mutant leaves infiltrated with *Psm* ES4326/*avrRpt2* at 2×10^3 cfu/cm^2.

plants never develop symptoms when infiltrated with this dose (46). The incompletely recessive phenotype is also reflected in the fact that *Psm* ES4326/*avrRpt2* grows almost as well in heterozygous *RPS2/rps2* leaves as it does in *rps2/rps2* leaves, whereas growth in *RPS2/RPS2* leaves is limited to ≈1% the level of that in *rps2/rps2* leaves (42, 45, 46).

Cloning the *Arabidopsis RPS2* Gene

The *Arabidopsis RPS2* gene was cloned by a map-based positional cloning strategy (43, 47). In our laboratory, the *RPS2* gene was first mapped to the bottom of chromosome IV (42, 46). Fine structure restriction fragment length polymorphism (RFLP) mapping showed that *RPS2* is closely linked to and

situated on the centromeric side of the anonymous RFLP marker *PG11* between the morphological markers *cer2* and *ap2* (Fig. 2*A*) (42, 43). Coincidentally, as described in a recent publication (48), the *Arabidopsis ABI1* gene was also found to be similarly situated with respect to *PG11*. To clone the *ABI1* gene, a set of overlapping cosmid clones that span *ABI1* had been identified, starting from and extending ≈200 kb to the centromeric side of *PG11*. J. Leung and J. Giraudat, Centre National de la Recherche Scientifique (CNRS), Gif-Sur-Yvette, France, kindly provided us with this set of cosmid clones and as illustrated in Fig. 2 *B* and *C*, additional RFLP analysis showed that *RPS2* maps to a 28- to 35-kb region within the 200-kb region that also contains *ABI1*.

To identify the *RPS2* gene within the 35-kb region to which it had been mapped, transcripts encoded within this region were cloned and sequenced and then compared to the corresponding sequences from four different *rps2* mutants (43). Specifically, six groups of cDNA clones encoded within the 35-kb region were identified as illustrated in Fig. 2*D*. Because mutations that alter the amino acid sequence were found in the transcripts corresponding to cDNA 2 from all four *rps2* mutant plants, we concluded that *RPS2* is the gene corresponding to this cDNA. The gene corresponding to cDNA 1 was recently identified as *ABI1* (48, 49). DNA blot analysis under low-stringency hybridization conditions suggested that *RPS2* is a single copy gene but that there may be several *RPS2*-related genes in *Arabidopsis* (43).

To verify that the gene identified as *RPS2* by DNA sequence analysis corresponds to the *RPS2* gene defined by genetic analysis, we carried out two types of genetic complementation analysis (43). We first used a transient assay for *RPS2* function that involves biolistic bombardment of *rps2* mutant leaves with a cloned *RPS2* cDNA. The transient assay works as follows: Briefly, *rps2* mutant leaves, preinfected with *P. syringae* carrying *avrRpt2*, are cobombarded with two plasmids: one contains a gene to be tested for complementation and the other contains the *Escherichia coli uidA* gene encoding β-glucuronidase (GUS). Both the test gene and the *uidA* gene are located downstream of the strong constitutive 35S promoter from cauliflower mosaic virus. If the test gene complements the *rps2* mutation, the transformed cells rapidly undergo programmed cell death in response to the *P. syringae* carrying *avrRpt2* and relatively little GUS activity accumulates. If the *rps2* mutation is not complemented, cell death does not occur and high levels of GUS activity accumulate. These differences in GUS activity are detected histochemically. In practice, a 35S cDNA 2 (*RPS2*) cDNA clone complemented four different *rps2* mutants that were tested, whereas 35S cDNA 1 (*ABI1*) and 35S cDNA 3 (Fig. 2*D*) did not complement.

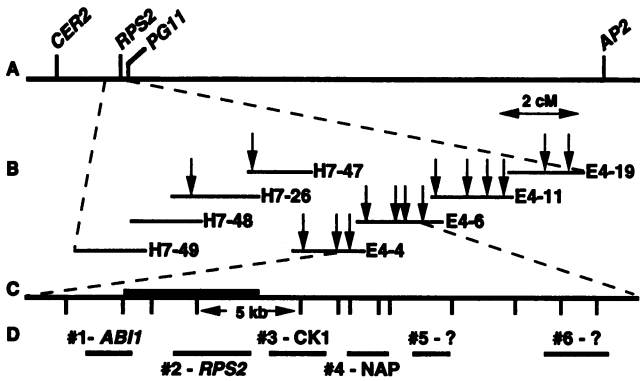


FIG. 2. Chromosome walk to clone *RPS2*. (*A*) Genetic map of the ≈15-centimorgan (cM) *cer2-ap2* region of chromosome IV containing *RPS2*. (*B*) Diagram showing alignment of cosmid clones (generously provided by J. Giraudat) around the *RPS2* locus. Vertical arrows represent relative positions of polymorphic restriction sites identified by using various cosmid clones as DNA probes for *Arabidopsis* DNA cut with a panel of restriction endonucleases. *RPS2* mapped to a 28- to 35-kb region spanned by cosmids E4-4 and E4-6. (*C*) *Eco*RI restriction map spanning the *RPS2* locus in the Columbia ecotype. The genomic region used for genetic complementation in transgenic plants is shown as a thicker line. (*D*) Relative positions of transcripts encoded and identified in the *RPS2* region. #, Number assigned to each transcript. Transcripts 3, 4, and 5 encode proteins with similarity to casein kinase I, (CK1), to nucleosome assembly protein I (NAP), and to no known proteins in GenBank, respectively.

We also carried out traditional genetic complementation analysis with *rps2* mutant plants used as recipients for a 5.9-kb *Sma* I/*Sac* I DNA fragment (thick line in Fig. 2*C*) containing the entire *RPS2* coding region as well as the entire intergenic upstream region between *ABI1* and *RPS2*. This DNA fragment complemented the *rps2* mutant phenotype of two different *rps2* mutants that were tested (43).

The *Arabidopsis RPS2* gene has been cloned independently by B. Staskawicz and colleagues (47).

Structure of RPS2 Protein

A schematic diagram of the 105-kDa *RPS2*-encoded protein is shown in Fig. 3. Four *rps2* mutations that were identified by DNA sequence analysis are described in the legend. RPS2 protein contains a nucleotide binding site (50) and 14 imperfect leucine-rich repeats (LRRs) (51). The nucleotide binding site, consisting of kinase 1a, -2, and -3a motifs, participates in ATP/GTP binding sites of various kinases, and its presence suggests that kinase activity is involved in RPS2 function (50). LRRs are 20- to 30-amino acid repeats of leucine-rich se-

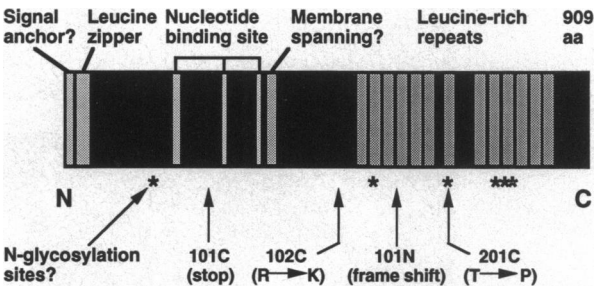


FIG. 3. Structure of RPS2 protein. The *RPS2* open reading frame consists of 2727 bp corresponding to a polypeptide of 909 amino acid residues with a relative molecular weight of 104,640. The approximate locations of mutations in the *rps2* alleles *rps2-101C, -102C, -201C*, and *-101N* are shown. The alleles *101C, 102C*, and *201C* contain single base mutations that change tryptophan 235 to a stop codon, arginine 476 to lysine, and threonine 668 to proline, respectively. Allele *101N* contains a 10-bp insertion after nucleotide 1744, which causes a shift in the *RPS2* reading frame. Motifs identified by computer analysis include a nucleotide binding site [kinase 1a, kinase 2, and kinase 3a motifs in amino acids 182–190, 258–262, and 330–335, respectively (50)] and 14 imperfect LRRs (amino acids 505–867; see Fig. 5; see also refs. 51 and 52 and references therein). The LRRs closer to the C terminus do not fit the consensus sequence very well compared to the ones closer to the N terminus, making it difficult to assign the C-terminal boundary of the LRR region with certainty. RPS2 also contains a region of heptad leucine repeats [leucine zipper; amino acids 31–52 (53, 54)] close to the N terminus. This region is predicted to form an amphipathic α-helix and could be involved in the formation of homo- or heterodimers or trimers. The computer program ALOM (55) predicts a membrane integrated region in the middle of the molecule (amino acids 340–356). Although RPS2 does not have a clear signal peptide sequence at the N terminus, it does have a relatively hydrophobic sequence (amino acids 7–22). The amino acid sequence surrounding the N-terminal hydrophobic region is more positively charged on the C-terminal side, making it more likely that the hydrophobic region functions as a signal anchor (type I) than as a signal peptide (56, 57). Finally, RPS2 contains six potential N-glycosylation sites at amino acid residues 158, 543, 666, 757, 778, and 787, which could be glycosylated if extracellular.

quence in which leucine and other aliphatic residues occur periodically. LRRs are contained in a wide variety of proteins and have been suggested to be involved in protein–protein interactions (51, 52). Two distinctive possibilities are that the RPS2 LRRs are either involved in ligand binding, like the LRR of gonadotropin receptors (58), or interact with other components of a signal transduction pathway, like the LRRs of yeast adenylate cyclase (CYR1) (59). RPS2 also contains a leucine zipper close to the N terminus, a predicted membrane integrated region in the middle of the molecule, and six potential N-glycosylation sites, which could be glycosylated if extracellular. The above features of RPS2 protein are described in more detail in the legend to Fig. 3.

Preliminary *in vitro* translation/translocation experiments indicated that RPS2 protein does not have a large noncytoplasmic portion and that it is not tightly bound to membrane by itself (F.K. and F.M.A., unpublished data). If RPS2 is a cytoplasmic receptor, either the *avrRpt2*-generated ligand must be a membrane-permeable signal or there must be a primary receptor that converts the *avrRpt2*-generated signal into a secondary cytoplasmic signal.

Remarkably, RPS2 protein is very similar to the product of the *Nicotiana tabacum N* gene, which confers resistance to infection by tobacco mosaic virus (60). The similarity between RPS2 and N is dispersed over the entire molecules, with approximately 25% identity and 50% similarity. In the N-terminal regions, there are patches of highly conserved portions including the kinase 1a motif. Interestingly, N protein does not appear to have a membrane integrated region even though the homology around the predicted RPS2 membrane integrated region is relatively high. Because it is reasonable to assume that the functionally and structurally related N and RPS2 proteins have a similar subcellular localization and/or topology, it appears likely that RPS2 functions as a cytoplasmic receptor.

Relationship Between RPS2 and N Proteins

What is the relationship between *RPS2* and *N*, on the one hand, and *PTO*, on the other, the two distinctive classes of resistance genes so far discovered that conform to gene-for-gene interactions? This is an important question because the gene-for-gene model predicts that all three resistance gene products are primary receptors for *avr*-generated signals. Another possibility, however, is that RPS2-like proteins are primary receptors and that PTO-like proteins are downstream components of a signal transduction pathway. This latter possibility is heretical to the traditional teaching of the gene-for-gene model, which postulates a single plant gene that can be mutated to block transduction of a particular *avr*-generated signal. In other words, because *PTO* corresponds to *avrPto* in a gene-for-gene manner, *PTO* must necessarily encode a protein that is the primary receptor of the *avrPto*-generated signal.

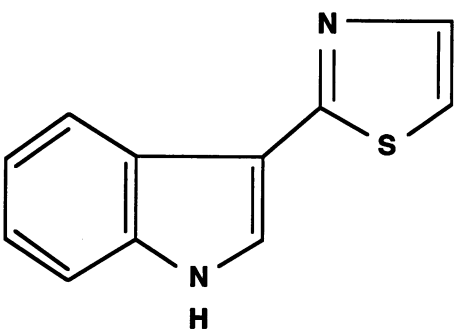
Within the past year, several reports have cast doubt on the validity of the simplest formulation of the gene-for-gene model. For example, a newly discovered gene (*PRF*) has been identified in tomato that appears to be involved in *avrPto* recognition in addition to *PTO* (61). Similarly, two tomato genes in addition to the resistance gene *Cf9* appear to be involved in the recognition of the fungal elicitor peptide encoded by the *C. fulvum avr9* gene (62). Finally, two additional genes are required for Mla_{12}-specified race-specific resistance to powdery mildew in barley (63). It may be common, therefore, that multiple plant-encoded proteins are required for the recognition of *avr*-generated signals. On the other hand, the overall similarity between the *Arabidopsis RPS2*- and tobacco *N*-encoded proteins supports the basic tenet of the gene-for-gene model, which postulates an array of related receptors that have evolved for the recognition of a wide variety of pathogens and that all feed into a common signal transduction cascade.

Arabidopsis Phytoalexin Mutants

In the isolation and characterization of *Arabidopsis rps2* mutants described above, we sought to identify mutants that lacked the ability to respond to *P. syringae* expressing the avirulence gene *avrRpt2*. This approach led to the isolation of mutants that most likely define the initial component of a signal transduction cascade leading to expression of defense responses that in turn render the plant resistant to pathogens carrying the avirulence gene. In contrast, in this section of the article, we describe how we used a particular defense response at the end of a signal transduction cascade to identify a series of mutants that affect resistance to pathogens.

The defense response that we chose to study is phytoalexin synthesis. Phytoalexins are small molecules that have antimicrobial activity and that are synthesized by plants in response to pathogen attack (64). Several lines of evidence support the hypothesis that phytoalexins are important components of plants' defensive arsenals. Phytoalexins are broad-spectrum antibiotics and inhibit the growth of fungal and bacterial phytopathogens *in vitro* (65). In many plant–pathogen systems, phytoalexins accumulate rapidly in response to avirulent pathogen races but not in response to virulent ones (1, 65). Introduction of a gene encoding stilbene synthase, a phytoalexin biosynthetic enzyme from grape, into tobacco conferred increased resistance against a fungal pathogen of tobacco (66). In contrast, recent work from the VanEtten laboratory (H. VanEtten, personal communication) has shown that mutants of *Nectria hematococca* that have lost the ability to detoxify the pea phytoalexin pisatin remain virulent but cause somewhat smaller disease lesions than wild-type fungi.

Arabidopsis produces a phytoalexin with the structure of 3-thiazole-2-yl-indole (27). This compound is commonly referred to as camalexin, because it was first identified as a phytoalexin produced by *Camelina sativa* (67). Camalexin appears to be the only phytoalexin that is produced in significant quantities by *Arabidopsis* (27). Infection of *Arabidopsis* by avirulent *P. syringae* bacteria induced camalexin biosynthesis, while infection by unrelated virulent *Xanthomonas campestris* bacteria did not (27). Camalexin was shown to inhibit the growth of a phytopathogenic fungus, *Cladosporium cucumerium*, and *P. syringae in vitro* (27).



Structure I

In our laboratory, the question of the role of camalexin in combating phytopathogens was approached by removing camalexin from the *Arabidopsis–P. syringae* interaction by genetic mutation and analyzing the effect on pathogen growth. In principle, camalexin could be important in rendering *Arabidopsis* resistant to pathogens that elicit gene-for-gene resistance responses like the *avrRpt2/RPS2* interaction, and/or it could be involved in restricting the growth of virulent pathogens. The question of how plants restrict the growth of virulent

pathogens is not often considered in plant pathology, but it is clear that plants do not necessarily allow unlimited growth of virulent pathogens. For example, when *Arabidopsis* leaves are infected with the virulent strain *Psm* ES4326 or *P. syringae* pv. *tomato* (*Pst*) DC3000, both the severity of symptoms and the maximum density of bacteria within the leaves depend on the concentration of the initial inoculum. When the initial inoculum is low, symptoms are less severe and maximum bacterial density is lower than when the initial inoculum is high (Fig. 4). This demonstrates that *Arabidopsis* is restricting the growth of these virulent strains, since, if it were not, the density of the bacteria would depend on only the capacity of leaves and not the inoculum concentration.

We examined the induction of camalexin by infection of *Arabidopsis* with various *P. syringae* strains (44). Camalexin accumulated to similar high levels in response to the virulent strain *Psm* ES4326 and the isogenic strains *Psm* ES4326/*avrRpt2* and *Psm* ES4326/*avrRpm1*, suggesting that the presence of avirulence genes had little effect on camelexin accumulation. However, camalexin accumulated more rapidly in response to strain *Pst* DC3000/*avrRpt2* than in response to the isogenic virulent strain *Pst* DC3000, suggesting that in the *Pst* DC3000 strain background, there is an effect of the avirulence gene in inducing camalexin accumulation. As described above, resistance of ecotype Columbia to *avrRpt2*-carrying strains requires the resistance gene *RPS2* (16, 29, 42, 45). To test whether the difference in camalexin induction between strains *Pst* DC3000/*avrRpt2* and *Pst* DC3000 was an *RPS2*-dependent response to the *avrRpt2* gene, camalexin accumulation in the Columbia ecotype *rps2* mutant *rps2-101C* (42) was examined. In *rps2-101C* plants, camalexin induction was similar in response to either *Pst* DC3000/*avrRpt2* or *Pst* DC3000, indicating that the effect of *avrRpt2* (in the *Pst* DC3000 strain background) on camalexin accumulation is mediated by *RPS2*. Evidently, camalexin levels are affected by multiple factors, including the genetic background of virulent strains and the presence of avirulence genes. Strains *Psm* ES4326, *Pst* DC3000, and *Psp NPS3121* (which is not a pathogen of *Arabidopsis*) and *E. coli* all displayed similar levels of camalexin sensitivity *in vitro*, suggesting that strains *Psm* ES4326 and *Pst* DC3000 are not particularly tolerant of camalexin.

Mutants of ecotype Columbia with defects in camalexin synthesis in response to pathogen attack were isolated by thin-layer chromatography (44). Approximately 7000 M2 generation plants from an ethyl methane sulfonate-mutagenized population were screened and three phytoalexin-deficient mutants were identified and named *pad1*, *pad2*, and *pad3*. In the *pad1* and *pad2* mutants, camalexin accumulated to approximately 30% and 10% of the levels reached in wild type, respectively. No camalexin was detected in the *pad3* mutant. The time course of camalexin accumulation in the *pad1* and *pad2* mutants was similar to that in Columbia. When wild-type and *pad* mutant plants were infected with *Psm* ES4326/*avrRpt2*, the reduction in camalexin levels in the three *pad* mutants relative to wild-type plants was as great as or greater than when plants were infected with *Psm* ES4326.

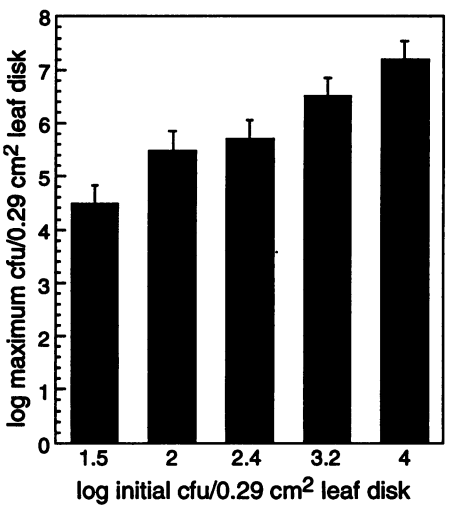


FIG. 4. Dependence of maximal density of *Psm* ES4326 on initial inoculum. *Psm* ES4326 was inoculated into *Arabidopsis* leaves at various concentrations. After 72 hr, bacterial density was determined. Each point represents the mean of six replicates; error bars show SD. In other experiments, we showed that bacterial density in infected leaves does not increase significantly after 72 hr regardless of the initial inoculum.

Genetic analyses demonstrated that all three *pad* mutations are recessive alleles of single nuclear genes (44). Complementation testing showed that they define three different genes, suggesting that additional *pad* loci remain to be identified. The CAPS mapping technique (68) was used to place the *pad* mutations on the *Arabidopsis* genetic map (44). The data showed that *pad1* and *pad2* were located on chromosome IV, between markers *AG* and *DHS*, while *pad3* was located on chromosome III, between *BGL2* and *gl-1*.

The growth of virulent strains and isogenic strains carrying cloned avirulence genes in wild-type and *pad* mutant plants was examined to determine whether the *pad* mutations caused defects in the ability of plants to resist infection by avirulent strains or to restrict the growth of virulent strains (44). Three isogenic pairs of strains were used in order to control for effects specific to particular avirulence genes or strain backgrounds. These were *Psm* ES4326 and *Psm* ES4326/*avrRpt2*, *Pst* DC3000 and *Pst* DC3000/*avrRpt2*, and *Psm* ES4326 and *Psm* ES4326/*avrRpm1*. A low inoculum was used in these studies so that there would be a possibility of observing deleterious effects of the *pad* mutations on limiting growth of virulent strains as well as strains carrying *avr* genes.

We found that none of the *pad* mutants was compromised for limiting the growth of any of the avirulent strains, strongly suggesting that camalexin is irrelevant for gene-for-gene mediated resistance in the *Arabidopsis–P. syringae* system (44). This resistance must be due to some other defense response(s). This finding illustrates the danger in assuming that because a particular response is observed during a resistant interaction it is involved in conferring resistance. Even though the avirulent strain *Pst* DC3000/*avrRpt2* induced more camalexin than the virulent strain *Pst* DC3000, loss of camalexin did not affect the plant's resistance to *Pst* DC3000/*avrRpt2*. Evidently, not all of the components of the defensive arsenals deployed in response to a particular pathogen are actually required for resistance to that pathogen.

Interestingly, the *pad1* and *pad2* mutants allowed significantly (10- to 50-fold) more growth of both virulent strains than wild-type plants did, although the *pad3* mutant limited their growth to the same extent as wild-type plants (44) (Table 1). This is a curious finding, since camalexin is undetectable in the *pad3* mutant, while the *pad1* and *pad2* mutants accumulate camalexin to 30% and 10% of wild-type levels, respectively. Since the apparently complete loss of camalexin in the *pad3* mutant did not result in increased pathogen growth, camalexin itself must not be required for limiting the growth of *Psm* ES4326 and *Pst* DC3000 *in planta*. This leaves the question of why the *pad1* and *pad2* mutations caused an increase in pathogen sensitivity.

One possible explanation is that phytoalexins are not required for limitation of *P. syringae* growth in *Arabidopsis*, but the *pad1* and *pad2* mutations exert pleiotropic effects on other defense responses, which are required for limiting pathogen growth. This could occur if *pad1* and *pad2* are lesions in genes encoding components of the signal transduction pathway leading to activation of plant defense responses. Preliminary experiments, however, demonstrated no difference in the activation of a variety of defense-related genes in *pad* mutants

Table 1. Phenotypes of *Arabidopsis* phytoalexin-deficient (*pad*) mutants

Genotype	Camalexin, % wt*	Virulent growth, log cfu/cm²†
Wild type‡	100	5.5
pad1	30	7.0
pad2	10	6.5
pad3	≤1	5.5

*Accumulation of camalexin in leaves 72 hr after infiltration.
†Titer of *P. syringae* 72 hr after infiltration.
‡Ecotype Columbia.

compared to wild-type plants (J.G. and F.M.A., unpublished data).

Another possible explanation for the pathogen growth phenotypes of the *pad* mutants is that phytoalexin is required for limitation of *Psm* ES4326 and *Pst* DC3000 growth. In this model, the increased pathogen growth in *pad1* and *pad2* is explained by the reduced camalexin levels in these mutants. The absence of a pathogen growth phenotype in the *pad3* mutant is explained by hypothesizing that the *pad3* mutation blocks the camalexin biosynthetic pathway at a point such that a precursor accumulates that is itself a phytoalexin (i.e., has antimicrobial activity). The presence of this intermediate compound limits pathogen growth. If this model is correct, the *pad1* and *pad2* mutations must affect camalexin biosynthesis at a point in the pathway so that any intermediates that accumulate do not have antimicrobial activity. When a fungal bioassay was used, no pathogen-inducible antimicrobial compounds were observed in the *pad3* mutant using the extraction and assay protocol that detects camalexin from wild-type plants. In preliminary experiments with different extraction protocols, no phytoalexins were observed in the *pad3* mutant, but such a molecule may eventually be found by using additional extraction and assay procedures (J.G. and F.M.A., unpublished data).

While our work has shown that camalexin is not required for resistance to avirulent *P. syringae* strains in *Arabidopsis*, it is still possible that camalexin will prove to be important for resistance to other avirulent pathogens. The similarity of camalexin to the commercial fungicide thiabendazole suggests that camalexin could play an important role in interactions with phytopathogenic fungi (67). Various species of fungi are known to infect *Arabidopsis*, and several gene-for-gene resistance responses have been identified in these systems (17–19, 21). Analysis of the effects of the *pad* mutations on these interactions should help to elucidate the role of camalexin in combating fungi.

Conclusions

The cloning of *avrRpt2* and *RPS2* and the isolation of defense-related mutants in a single *Arabidopsis* ecotype have established a completely isogenic plant–microbe interaction model in which the pathogens differ from each other only in the expression of an avirulence gene and the hosts differ only in the expression of a corresponding resistance gene or another gene involved in the defense response. In the future, we will be able to use these genetic tools to systematically dissect the plant response to pathogen attack. More generally, the fact that an *Arabidopsis* bacterial pathogen had not even been described when we initiated the development of the *Arabidopsis–P. syringae* model attests to the utility of *Arabidopsis* for the study of complex problems in plant biology.

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