Arabidopsis Mutations at the *RPS2* Locus Result in Loss of Resistance to *Pseudomonas syringae* Strains Expressing the Avirulence Gene *avrRpt2*

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We isolated and characterized two *Arabidopsis thaliana* mutants that fail to mount a hypersensitive defense response (HR) when infiltrated with phytopathogenic *Pseudomonas* strains carrying the avirulence (*avr*) gene *avrRpt2* but still mount an HR when infiltrated with strains carrying other *avr* genes. One of these mutants was isolated using a method we developed that enriches for *Arabidopsis* seedlings that survive vacuum-infiltration with a bacterial strain carrying an *avr* gene. Genetic analysis showed that the phenotypes of both mutants resulted from mutations at a single locus, *RPS2*. In contrast to the wild type, both *rps2* mutants failed to limit the growth of *Pseudomonas* strains carrying *avrRpt2*. Heterozygous *RPS2/rps2* plants displayed a phenotype intermediate between those of *RPS2/RPS2* and *rps2/rps2* homozygotes. These experiments show that the wild-type allele at the *rps2* locus, *RPS2*, encodes a component of a signal transduction pathway that responds to a signal generated by *avrRpt2* and that *RPS2* is required for the elicitation of an HR. *RPS2* was mapped near the restriction fragment length polymorphism marker PG11 on chromosome IV.

Additional keywords: defense response, gene-for-gene, hypersensitive response.

Plants employ a variety of defensive strategies to combat pathogens (Keen 1992; Lamb *et al.* 1989). One defense response, the so-called hypersensitive response (HR), involves rapid localized necrosis of infected tissue (Klement 1982). In several host-pathogen interactions that have been studied in detail, genetic analysis has revealed a gene-for-gene correspondence between a particular avirulence (*avr*) gene in an avirulent pathogen that elicits an HR and a particular resistance gene in the host (Crute *et al.* 1985; Ellingboe 1981; Flor 1971; Keen and Staskawicz 1988). A simple model that explains the gene-for-gene correspondence of *avr* and resistance genes is that resistance genes encode receptors for molecular signals generated by *avr* genes. Signal transduction pathway(s) then carry the signal to a set of target molecules that initiate the HR and other host defenses (Gabriel and Rolfe 1990). Despite this simple predictive model, the molecular basis of the *avr*–resistance gene interaction is still unknown.

The gene-for-gene model has been strongly supported by the molecular cloning of bacterial and fungal *avr* genes that confer an avirulent phenotype when transferred to an otherwise virulent strain (Crute *et al.* 1985; Ellingboe 1981; Flor 1971; Keen and Staskawicz 1988). In a limited number of cases, the specific signals that elicit an HR have been identified. For example, the *avr9* gene of *Cladosporium fulvum* encodes a peptide elicitor (van den Ackerveken *et al.* 1992), the *avrD* locus of *Pseudomonas syringae* pv. *glycinea* encodes proteins that elaborate a low molecular weight elicitor (Keen *et al.* 1990; Keen and Buzzell 1991), and the *hrpN* gene from *Erwinia amylovora* encodes a proteinaceous elicitor (Wei *et al.* 1992).

Compared to *avr* genes and the signals they generate, considerably less is known about plant resistance genes that correspond to specific *avr*-generated elicitors. To initiate a study of plant resistance genes in a system that is amenable to molecular genetic analysis, we and others have developed a model pathogenesis system that involves the infection of *Arabidopsis thaliana* with pathogenic bacteria and fungi (Davis *et al.* 1991; Debener *et al.* 1991; Dong *et al.* 1991; Koch and Slusarenko 1990; Simpson and Johnson 1990; Tsuji *et al.* 1990; Whalen *et al.* 1991). In previous work from our laboratory, the virulent *P. syringae* pv. *maculicola* strain ES4326 was shown to multiply and to cause the formation of water-soaked lesions when infiltrated into *Arabidopsis* leaves (Davis *et al.* 1991; Dong *et al.* 1991). In contrast, the avirulent *P. syringae* pv. *tomato* strain MM1065 failed to proliferate in *Arabidopsis* leaves and caused the appearance of mildly chlorotic spotted dry lesions (Dong *et al.* 1991; Whalen *et al.* 1991). An *avr* gene, *avrRpt2*, was cloned from strain MM1065 on the basis that when it was transferred to *P. s.* pv. *maculicola* ES4326, the transconjugant, from here on termed ES4326/*avrRpt2*, elicited a strong HR-like response within 16 hr and multiplied 50- to 100-fold less than *P. s.* pv. *maculicola* ES4326 in *Arabidopsis* leaves (Dong *et al.* 1991; Whalen *et al.* 1991).

In work from other laboratories, natural variation in the resistance response to *P. syringae* strains carrying *avrRpt2* (Kunkel *et al.*, *in press*) and *avrRpm1* (Debener *et al.* 1991) was observed among different *Arabidopsis* ecotypes. Genetic analysis of these interecotype differences

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showed that single genetic loci were most likely responsible for the different host responses (Debener *et al.* 1991; Kunkel *et al., in press*). In the experiments reported in this paper, we describe the isolation and characterization of two *Arabidopsis* mutants that fail to mount an HR when infiltrated with *P. syringae* strains carrying *avrRpt2*. These mutants have the phenotype expected of *Arabidopsis* plants that carry mutations in a resistance gene that corresponds specifically to *avrRpt2*. We chose to screen for resistance gene mutants in a particular *Arabidopsis* ecotype rather than looking for natural variation among *Arabidopsis* ecotypes because we wanted to avoid a potential problem caused by differences in the genetic backgrounds of different ecotypes. *Arabidopsis* mutants with a similar phenotype have also been isolated by Kunkel *et al.* (*in press*).

RESULTS

Isolation of *Arabidopsis* mutants that fail to mount an HR in response to *avrRpt2*.

Our strategy for isolating *Arabidopsis* resistance gene mutants involved the use of isogenic pairs of *P. syringae* strains that differed only in the expression of a single open reading frame corresponding to the avirulence gene *avrRpt2* carried on plasmid pLH12 (Whalen *et al.* 1991; Innes *et al., in press*). In comparison to using an uncharacterized strain that might express two or more *avr* genes, we reasoned that the use of *avrRpt2* expressed in an otherwise virulent strain would increase the chances of isolating mutations in a resistance gene corresponding to *avrRpt2*.

Two different methods were used to identify *Arabidopsis* mutants that respond aberrantly to *avrRpt2*. First, as described in Methods, 4- to 6-wk-old individual ethylmethyl-sulfonate-mutagenized M_2 *Arabidopsis* plants were hand-infiltrated with ES4326/*avrRpt2* at a titer of approximately 4×10^4 cells for each square centimeter of leaf area and then scored for the absence of a macroscopic HR. Among approximately 3,000 infiltrated plants, one plant failed to mount an HR 22 hr after infiltration. The presumptive mutant locus in this plant was named *rps2* (resistance to *P. syringae*), and the mutation at the *rps2* locus in this mutant plant was given the allele number 101C, where "C" designates the *Arabidopsis* Columbia ecotype.

A second presumptive *rps2* mutant allele (*rps2-102C*) was isolated using a method that was based on the observation that even very high doses (10^6 cells/cm^2) of the bean pathogen *P. syringae* pv. *phaseolicola* strain NPS3121 failed to elicit disease symptoms or a visible HR on *Arabidopsis* ecotype Columbia leaves, whereas NPS3121/*avrRpt2* elicited a strong HR (M. Mindrinos and F. Ausubel, *unpublished*). We reasoned that if NPS3121/*avrRpt2* were vacuum-infiltrated into an entire seedling, it might elicit an HR-like response throughout the seedling and kill it. On the other hand, a mutant that did not respond to a signal generated by *avrRpt2* might survive the infiltration procedure because *P. s.* pv. *phaseolicola* NPS3121 is a nonhost pathogen. As shown in Figure 1, when 2-wk-old *Arabidopsis* seedlings growing on agar in a petri dish were vacuum-infiltrated with *P. s.* pv. *phaseolicola* NPS3121, about 90–95% of the seedlings survived, whereas only 5–10% of the seedlings survived infiltration with NPS3121/*avrRpt2*.

Approximately 4,000 ethylmethyl-sulfonate-mutagenized M_2 seedlings were vacuum-infiltrated with 8×10^6 cells/ml of NPS3121/*avrRpt2*. The survivors were transplanted to soil 5 days after infiltration, grown to maturity, and tested for their ability to mount an HR following hand-infiltration with ES4326/*avrRpt2*. Among 200 survivors, one plant (*rps2-102C*) failed to show an HR.

When rps2-101C and rps2-102C were allowed to self, all of the progeny in subsequent generations (M_3 and M_4) that were tested displayed the same mutant phenotype as rps2-101C and rps2-102C, indicating that the initial mutant isolates were homozygous. To separate the *rps2-101C* and *rps2-102C* mutations from other unlinked mutations, both rps2 mutants were backcrossed to wild-type Columbia plants, F_1 generation plants were selfed, and homozygous *rps2*/*rps2* F_2 generation plants were selected. This backcrossing procedure was repeated. Both the first and second backcross generation homozygous *rps2*/*rps2* plants displayed the same phenotypes in interaction with *Pseudomonas* strains as the original M_2 generation mutants. The two rps2 mutants are not likely to be siblings because they displayed different morphological mutant phenotypes which segregated away from the *rps2*/*rps2* phenotypes in backcrossing to the wild type. Homozygous *rps2-101C*/*rps2-101C* and *rps2-102C*/*rps2-102C* plants that did not display the unlinked morphological mutant phenotypes and that did not segregate these phenotypes in subsequent generations were selected for further study.

Symptom development in rps2 mutants.

Figure 2 shows the symptoms elicited in wild-type and homozygous *rps2-101C*/*rps2-101C* mutant leaves (rps2-101C leaves) infiltrated with *P. s.* pv. *maculicola* ES4326 (the leaves on the right of each panel) and with ES4326/*avrRpt2* (the middle leaves of each panel). Wild-type and rps2-101C leaves were also infiltrated with *P. s.* pv. *maculicola* ES4326 carrying two additional *avr* genes, *avrRpm1*, (Debener *et al.* 1991) (the leaves on the left of each panel), and *avrB* (Staskawicz *et al.* 1987) (data not shown). Both ES4326/*avrRpm1* and ES4326/*avrB* elicit an HR on wild-type *Arabidopsis* leaves. These latter *avr* genes were tested to distinguish mutants that have a general defect in their ability to mount an HR from mutants that specifically fail to mount an HR in response to a signal generated by *avrRpt2*.

A high dose (4×10^4 cells/cm^2) of ES4326/*avrRpt2*, ES4326/*avrRpm1* (Fig. 2B), or ES4326/*avr*B (not shown) was required to elicit a visible HR on wild-type plants, which appeared within 16–24 hr. This same dose of *P. s.* pv. *maculicola* ES4326 had no visible effect on wild-type leaves within 24 hr (Fig. 2B); however, disease symptoms developed on wild-type leaves after 24–48 hr (not shown). When infiltrated at a lower dose ($2–4 \times 10^3$ cells/cm^2), ES4326/*avrRpt2*, ES4326/*avrRpm1* (Fig. 2A), or ES4326/*avrB* (not shown) elicited no visible symptoms on wild-type leaves over the course of several days, whereas infiltrating this lower dose of *P. s.* pv. *maculicola* ES4326 (Fig. 2A) caused the appearance of a yellow chlorotic lesion within 2–3 days. Importantly, a high dose (2×10^4 cells/

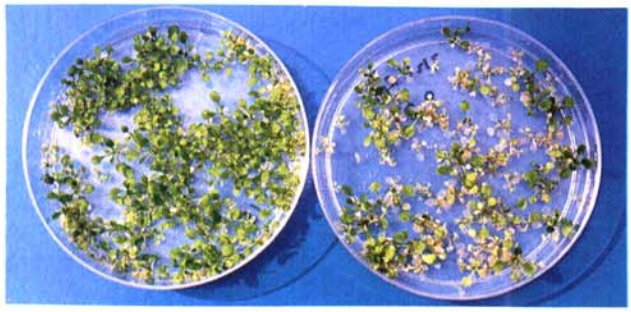

Fig. 1. Killing of *Arabidopsis* seedlings by infiltrating *Pseudomonas syringae* pv. *phaseolicola* NPS3121/*avrRpt2*. Ten-day-old seedlings were vacuum-infiltrated with *P. s.* pv. *phaseolicola* NPS3121 (left plate) or *P. s.* pv. *phaseolicola* NPS3121/*avrRpt2* (right plate) at a titer of 8×10^7 cells/ml. The photograph was taken 3 days after infiltration.

cm^2) of ES4326/*avrRpt2* (Fig. 2E) did not elicit an HR on rps2-101C leaves, whereas ES4326/*avrRpm1* (Fig. 2E) or ES4326/*avrB* (not shown) elicited an HR on rps2-101C leaves similar to that elicited on the wild type. Conversely, rps2-101C developed disease symptoms with chlorotic lesions 2–3 days after infiltration of either *P. s.* pv. *maculicola* ES4326 or ES4326/*avrRpt2* (Fig. 2D), but not following infiltration of ES4326/*avrRpm1* (Fig. 2D) or ES4326/*avrB* (not shown), at a dose of 4×10^3 cells/cm^2. These observations indicate that the inability of rps2-101C to mount an HR or suppress symptom development in response to ES4326/*avrRpt2* is not due to a general defect in the ability of rps2-101C to undergo localized cell death. Rather, the defect in rps2-101C appears to be specific in its ability to respond to strains carrying *avrRpt2*.

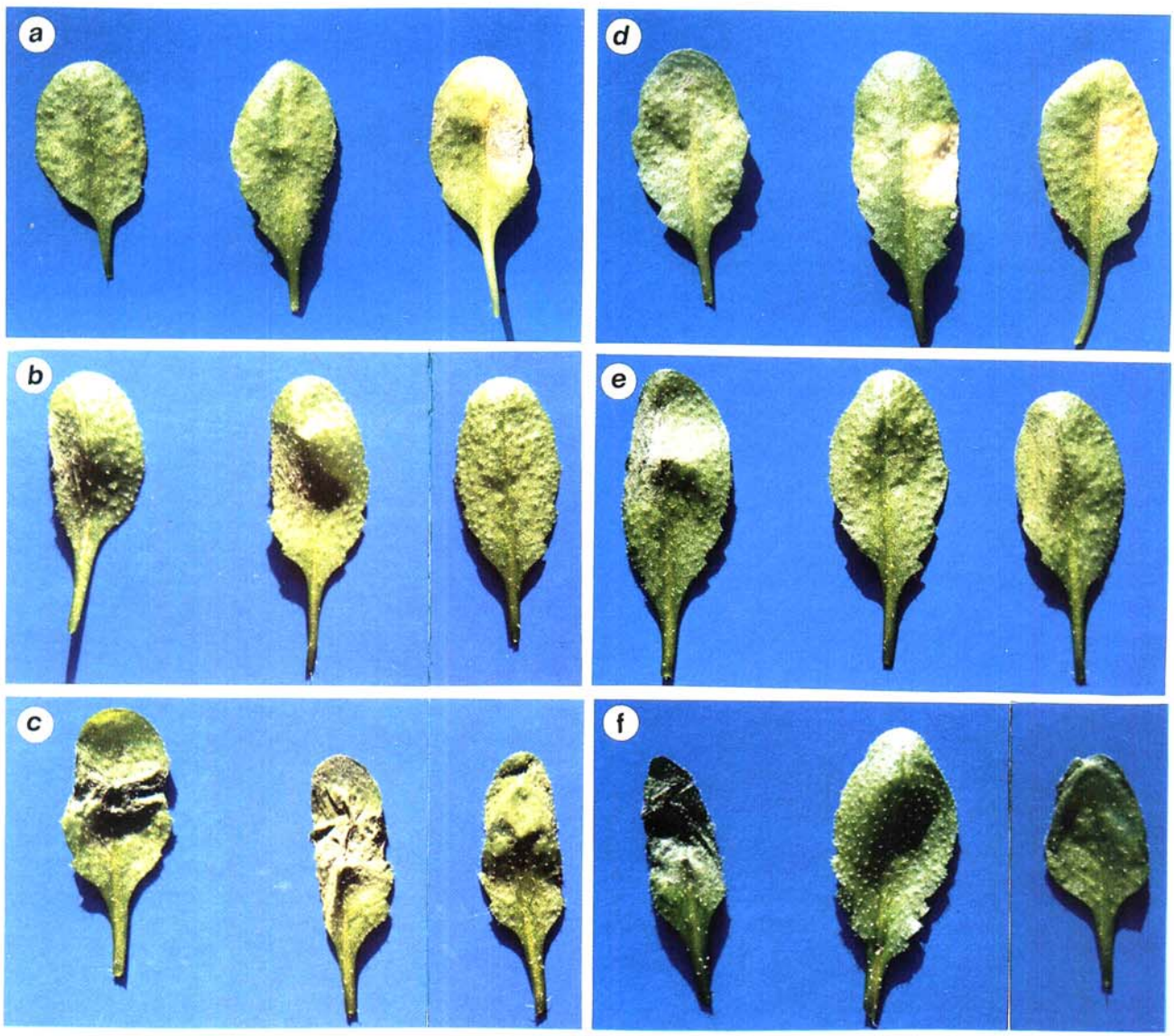

Fig. 2. Symptoms elicited by virulent and avirulent *Pseudomonas syringae* strains infiltrated into *Arabidopsis* leaves. **A, B, and C,** Wild-type (*RPS2*/*RPS2*) Columbia and **D, E, and F,** *rps2-101C*/*rps2-101C* leaves were infiltrated with *P. s.* pv. *maculicola* ES4326/*avrRpm1* (leaves on the left in each panel), *P. s.* pv. *maculicola* ES4326/*avrRpt2* (leaves in the middle) or *P. s.* pv. *maculicola* ES4326 (leaves on the right in each panel). Leaves in **A and D** were infiltrated with 2×10^3 cells/cm^2 leaf area and photographed 72 hr post infiltration. Leaves in **B and E** were infiltrated with 2×10^4 cells/cm^2 and photographed 24 hr post infiltration. Leaves in **C and F** were infiltrated with 2×10^5 cells/cm^2 and photographed 24 hr post infiltration.

In addition to the results shown in Figure 2 obtained with ES4326/*avrRpt2*, we found that NPS3121/*avrRpt2*, which elicited a strong HR on wild-type leaves, elicited no visible symptoms on rps2-101C (*data not shown*). This was the expected result since *P. s.* pv. *phaseolicola* NPS3121 elicits no symptoms on wild-type leaves. The failure of NPS3121/*avrRpt2* to elicit symptoms on the rps2-101C also demonstrates that the failure of ES4326/*avrRpt2* to elicit an HR on rps2-101C is specific to *avrRpt2* and does not depend on a specific *Pseudomonas* host strain.

Fig. 3. Development of disease symptoms in *rps2-101C/rps2-101C* plants in response to *Pseudomonas syringae* pv. *maculicola* ES4326/*avrRpt2*. Wild-type Columbia (two plants on the left) and *rps2-101C/rps2-101C* (two plants on the right) were dipped in a suspension of *P. s.* pv. *maculicola* ES4326/*avrRpt2* (2×10^6 cells/ml) in 10 mM $MgCl_2$ and 0.01% Silwet L77. The photograph was taken 4 days after inoculation.

Although rps2-101C failed to mount an HR following infiltration of a moderate dose of ES4326/*avrRpt2*, rps2-101C mutant leaves responded differently to *P. s.* pv. *maculicola* ES4326 and ES4326/*avrRpt2* when doses greater than 2×10^5 cells/cm^2 were infiltrated. When entire wild-type leaves were infiltrated with *P. s.* pv. *maculicola* ES4326 at a titer of 2×10^5 cells/cm^2, an HR-like necrotic response occurred within 24 hr that was indistinguishable from the HR response elicited by similar doses of ES4326/*avrRpt2* or ES4326/*avrRpm1* (Fig. 2C). However, ES4326/*avrRpt2* did not cause leaf collapse in rps2-101C when infiltrated at a titer of 2×10^5 cells/cm^2, even though *P. s.* pv. *maculicola* ES4326 and ES4326/*avrRpm1* caused collapse as in the wild type (Fig. 2F).

In addition to observing the response of the rps2 mutant leaves to manual infiltration, symptom development was also monitored after dipping leaves in bacterial suspensions containing the wetting agent Silwet L77, which facilitates the formation of localized disease lesions (Whalen *et al.* 1991). Distinct chlorotic lesions appeared 48 hr after wild-type and rps2-101C plants were dipped in 2×10^6 cells/ml of *P. s.* pv. *maculicola* ES4326 (*data not shown*), whereas no visible symptoms developed on wild-type leaves dipped in the same titer of ES4326/*avrRpt2* (Fig. 3, plants on the left). In contrast to wild-type leaves, rps2-101C leaves developed disease symptoms 48 hr after dipping in ES4326/*avrRpt2* (Fig. 3, plants on the right).

All of the experiments in this section were also carried out with rps2-102C with indistinguishable results (*data not shown*).

Growth of bacterial strains in rps2 mutant leaves.

Growth of bacterial strains carrying different *avr* genes in rps2-101C and rps2-102C leaves supports the conclusion that the two rps2 mutants are specifically defective in their

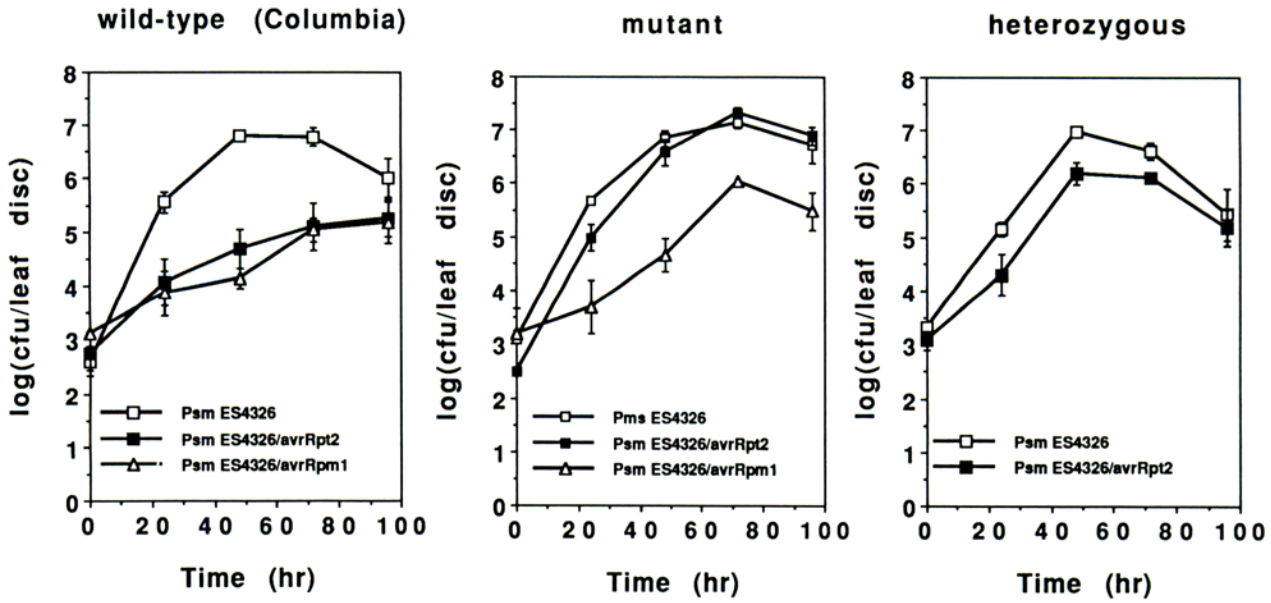

Fig. 4. Bacterial growth in wild-type (*RPS2/RPS2*), mutant (*rps2-101C/rps2-101C*), and heterozygous (*RPS2/rps2-101C*) *Arabidopsis* plants. Leaves were infiltrated with 4×10^3 cells/cm^2 of *Pseudomonas syringae* pv. *maculicola* ES4326, *P. s.* pv. *maculicola* ES4326/*avrRpt2*, or *P. s.* pv. *maculicola* ES4326/*avrRpm1*. The number of viable bacterial cells in each leaf punch was determined as described in Methods. Each point represents the mean of the logarithm of the number of bacteria in six leaf disks. Vertical bars indicate standard deviations. All growth curves were measured in at least two independent experiments.

ability to respond to a signal generated by *avrRpt2*. Wild-type and mutant leaves were infiltrated with ~4 × 10^3 cells/cm^2 of *P. s.* pv. *maculicola* ES4326, ES4326/*avrRpt2*, or ES4326/*avrRpm1*. As shown in Figure 4, *P. s.* pv. *maculicola* ES4326 multiplied up to 10^4-fold in wild-type leaves over the course of 3–5 days, whereas ES4326/*avrRpt2* grew 50- to 100-fold less than *P. s.* pv. *maculicola* ES4326. ES4326/*avrRpm1* also grew 50- to 100-fold less than *P. s.* pv. *maculicola* ES4326. The presence of *avrRpm1* in *P. s.* pv. *maculicola* ES4326 had less effect on growth

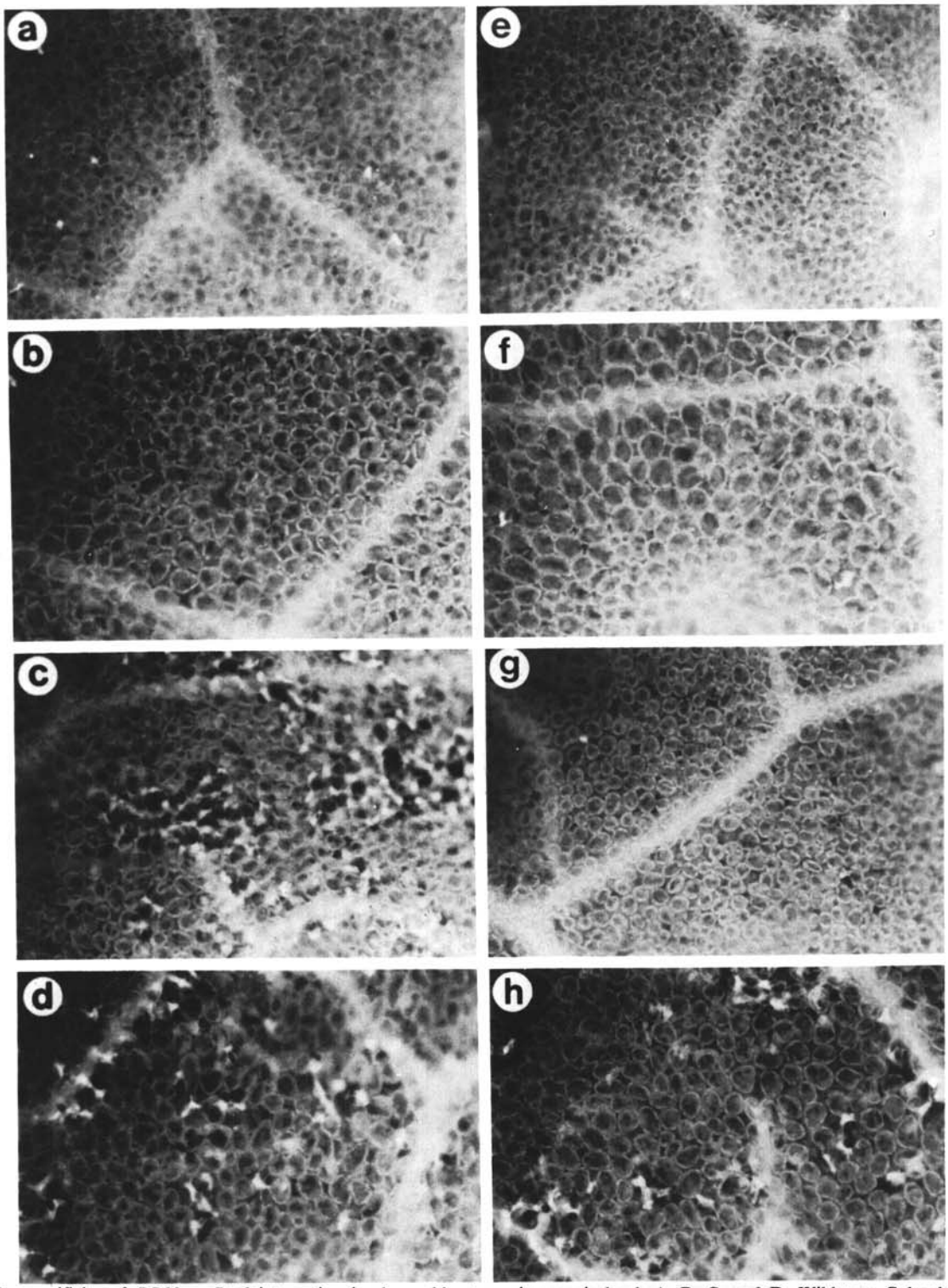

Fig. 5. The specificity of *RPS2-avrRpt2* interaction is observable at a microscopic level. **A, B, C, and D,** Wild-type Columbia and **E, F, G, and H,** *rps2-101C/rps2-101C* leaves were infiltrated with 10 mM $MgCl_2$ in **A and E**, *P. s.* pv. *maculicola* ES4326 in **B and F**, *P. s.* pv. *maculicola* ES4326/*avrRpt2* in **C and G**, or *P. s.* pv. *maculicola* ES4326/*avrRpm1* in **D and H**. The infiltration dose was 4 × 10^3 cells/cm^2. Leaves were fixed as described in Methods 24 hr after infiltration. The width of each panel is ~1 mm.

than it did in *P. s.* pv. *maculicola* M4 as reported by Debener *et al.* (1991). In contrast to the growth in wild-type leaves, both *P. s.* pv. *maculicola* ES4326 and ES4326/*avrRpt2* grew 10^4-fold in rps2-101C (Fig. 4) and rps2-102C (*data not shown*), whereas the growth of ES4326/*avrRpm1* was limited to the same degree in rps2-101C leaves as it was in wild-type leaves.

Evidence of HR deficiency of rps2-101C at a microscopic level.

In addition to monitoring the macroscopic appearance of HR lesions and monitoring bacterial growth in infected leaves, we sought a microscopic assay to monitor the response of *Arabidopsis* to *avrRpt2*. In potato, individual plant cells undergoing an HR have been detected in fixed leaves by the accumulation of an ethanol-nonextractable autofluorescence associated with cell wall regions (Schroder *et al.* 1992). As shown in Figure 5, a similar assay performed in our system showed that wild-type leaves appeared unaffected 24 hr after infiltration with *P. s.* pv. *maculicola* ES4326 (Fig. 5B) at a titer of 4×10^3 cells/cm^2 compared with control leaves infiltrated with 10 mM $MgCl_2$ (Fig. 5A). In contrast, a bright yellow fluorescence was observed associated with the peripheral region of patches of cells following infiltration with ES4326/*avrRpt2* (Fig. 5C) or ES4326/*avrRpm1* (Fig. 5D) at this same dose, which is too low a dose to elicit the appearance of a macroscopic HR. Moreover, in those cells surrounded by the yellow fluorescence, there was a clear loss of intracellular structures, most likely an indication of cell death. The amount of autofluorescence observed increased with increasing numbers of ES4326/*avrRpt2* cells infiltrated and was visible as early as 7 hr after infiltration.

In contrast to the wild-type plants (Fig. 5A–D), rps2-101C appeared unaffected by infiltration with either ES4326/*avrRpt2* (Fig. 5G) or *P. s.* pv. *maculicola* ES4326 (Fig. 5F), both of which looked similar to rps2-101C leaves infiltrated with 10 mM $MgCl_2$ (Fig. 5E). However, rps2-101C (Fig. 5H) responded to infiltration with ES4326/*avrRpm1* in the same manner as the wild type (Fig. 5D). Thus at both the macroscopic and microscopic levels, clear phenotypic differences could be observed between the response of wild-type plants and rps2-101C to ES4326/*avrRpt2*.

Genetic analysis of rps2 mutants.

Table 1 shows the results of scoring F_2 generation plants from three different crosses, *rps2-101C/rps2-101C* × *RPS2/RPS2* (Columbia, Col-0); *rps2-102C/rps2-102C* × *RPS2/RPS2* (Col-0); *RPS2/RPS2* (Landsberg *erecta*; La-*er*) × *rps2-101C/rps2-101C*, for the appearance of an HR following infiltration of ES4326/*avrRpt2* at a titer of ~4×10^4 cells/cm^2. These data are most consistent with a 3:1 segregation of HR to lack of HR and indicate that the mutations in rps2-101C and rps2-102C that are responsible for the phenotypes described above segregate as expected for single nuclear genetic loci.

Analysis of heterozygous *RPS2/rps2* plants showed that they had a phenotype intermediate between those of *RPS2/RPS2* and *rps2/rps2* homozygotes. First, the HR in heterozygous plants (from crossing *rps2-101C/rps2-101C* with wild-type Col-0 or La-*er*) required 20–24 hr to develop compared with 16 hr for wild-type plants. Second, a titer of ~1×10^5 cells/cm^2 of ES4326/*avrRpt2* was often required to elicit an HR in the heterozygous plants compared with a titer of ~1×10^4 cells/cm^2 for the wild type. Third, when the F_2 progeny in crosses 1 and 3 in Table 1 were scored for an HR, among the F_2 plants that gave an HR, two-thirds (122 of 188 and 66 of 99, respectively) showed weaker and delayed HR symptoms relative to the remaining F_2 plants (66 of 188 and 33 of 99), which showed a strong HR similar to those seen in wild-type plants. Fourth, the amount of autofluorescence observed in heterozygous plants was intermediate between that in wild-type and homozygous mutant plants. Fifth, as shown in Figure 4, when infiltrated at a titer of ~4×10^3 cells/cm^2, the growth of ES4326/*avrRpt2* was not restricted as much in heterozygous leaves as in wild-type leaves. Finally, disease symptoms sometimes developed in heterozygous leaves after infiltration with a relatively low titer of ES4326/*avrRpt2* (~4×10^3 cells/cm^2).

In addition to crossing the two ecotype Columbia *rps2/rps2* mutants with *RPS2/RPS2* plants, *rps2-101C/rps2-101C* was crossed with *rps2-102C/rps2-102C*, and *rps2-101C/rps2-101C* was crossed with an additional independently isolated *Arabidopsis* mutant that failed to mount an HR in response to *P. syringae* strains carrying *avrRpt2*. This mutant, rps2-201C, was also isolated in the Columbia ecotype (Kunkel *et al.*, *in press*). F_1 plants generated from the cross *rps2-101C/rps2-101C* × *rps2-102C/rps2-102C* failed to give an HR when infiltrated with ES4326/*avrRpt2*. As shown in Table 1, none of the 253 F_2 progeny plants tested mounted an HR. These data suggest that *rps2-101C* and *rps2-102C* are allelic. Equivalent results

Table 1. Genetic analysis of *rps2-101C* and *rps2-102C*

Cross[a] (recipient × donor) Generation	No. of plants displaying symptom[b] after infiltration with ES4326/*avrRpt2* HR$^+$	HR$^-$	$\chi^2_{3:1}$	*P*
rps2-101C × Col-0				
F_1	53[c]	0		
F_2	188[d]	56	0.55	0.46
rps2-102C × Col-0				
F_1	4[c]	0		
F_2	184[d]	59	0.067	0.80
La-*er* × *rps2-101C*				
F_1	24[c]	0		
F_2	99[d]	45	3.0	0.08
rps2-101C × *rps2-102C*				
F_1	0	6		
F_2	0	253	...	...
rps2-101C × *rps2-201C*				
F_1	0	9		
F_2	0	254	...	...

[a] All of the rps2 mutants used in these crosses were homozygous for the mutant *rps2* allele.
[b] Plants displayed a hypersensitive reponse (HR$^+$) or no hypersensitive response (HR$^-$).
[c] HR delayed. A delayed HR appeared 20–24 hr post infiltration, whereas a normal HR appeared 16 hr post infiltration.
[d] Approximately two-thirds of the plants showed a delayed HR (see text).

were obtained in the *rps2-101C/rps2-101C* × *rps2-201C/rps2-201C* cross (Table 1).

RPS2 maps to chromosome IV.

A polymerase chain reaction (PCR)-based mapping strategy developed recently in our laboratory (Konieczny and Ausubel, *in press*) was used to map *rps2-101C* to the bottom of chromosome IV (Fig. 6). Progeny from 104 individual selfed F_2 plants from the La-*er* × *rps2-101C/rps2-101C* cross shown in Table 1 were collected and approximately 20 plants from each of these F_3 families were infiltrated with 4×10^4 to 1×10^5 cells/cm^2 of ES4326/*avrRpt2*. If all or none of the plants in a particular F_3 family displayed an HR, that family was scored as *RPS2/RPS2* or *rps2-101C/rps2-101C*, respectively. If some F_3 plants in a particular family displayed an HR whereas the others did not, the family was scored as heterozygous. DNA was isolated from 20–30 F_3 progeny derived from each of the 104 F_2 plants. Initially, only PCR-based markers on chromosome IV (*GA1, AG1,* PG11, and *DHS1*) were tested, because an independently isolated *Arabidopsis* mutant with a similar phenotype had been shown to map on chromosome IV (Kunkel *et al., in press*). As shown in Table 2, we found that *rps2-101C* is tightly linked to the PCR marker PG11 (1% recombination). No linkage of *RPS2* was found to PCR-based markers distributed on the other four chromosomes (*data not shown*).

Two restriction fragment length polymorphism (RFLP) markers linked to PG11, 19838 and 17340, were also tested for their linkage to *rps2-101C*. DNA isolated as described above from 20–30 F_3 progeny derived from each of the 104 F_2 plants was digested with *Xba*I and *Hin*dIII, blotted, and probed with ^{32}P-labeled 19838 and 17340 DNA. The number of recombination events between *rps2-101C* and each of these RFLP markers is listed in Table 2.

Figure 6 shows the map position of *rps2-101C* with respect to the chromosome IV markers listed in Table 2. The MAPMAKER software (Lander *et al.* 1987) was used to determine the order of markers and map distances.

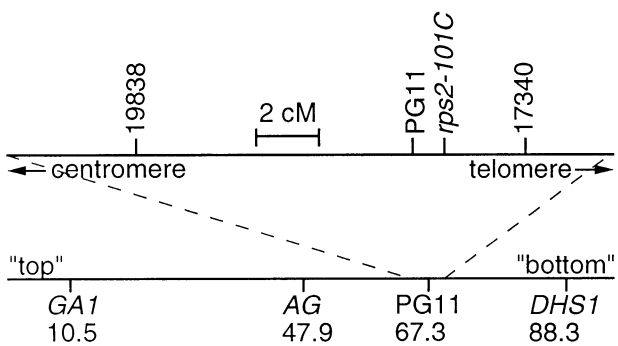

Fig. 6. Map position of *RPS2* on chromosome IV. The markers are described in Table 2. Map positions of *GA1, AG,* PG11, and *DHS1*, shown below their names in centimorgans, were obtained from version 1.3 of AAtDB (Cherry *et al.* 1992). Our mapping data place RFLP marker 17340 to the telomere side of PG11 in contrast to the position reported in the unified *Arabidopsis* genetic map in version 3.1 of AAtDB.

DISCUSSION

Enriching for *Arabidopsis* mutants that cannot mount an HR.

The most commonly used method of inoculating plants with a bacterial pathogen is to use a syringe without a needle to force a small amount of a bacterial suspension into stomatal openings. Although reliable and effective, hand-infiltration of individual plants is time-consuming. A heavily mutagenized population of *Arabidopsis* M_2 plants contains a mutation in a particular gene at a frequency of approximately one in 2,000-4,000 seedlings (U. Hanfstingl and F. M. Ausubel, *unpublished*), and a skilled worker can hand-inoculate and score approximately 200–300 plants per day. Therefore, identification of many *Arabidopsis* mutants that cannot mount an HR using a hand-infiltration procedure is an arduous task.

To overcome the tedium of hand-inoculating individual leaves, a non-ionic detergent has been used as a surfactant, which makes it possible to inoculate leaves simply by dipping them in a bacterial suspension (Whalen *et al.* 1991). An alternative procedure for mass inoculation of *Arabidopsis* plants involves vacuum-infiltration of densely sown mature plants growing on soil in small flats (M. Mindrinos, J. Glazebrook, and F. Ausubel, *unpublished*). In this procedure, the flats are inverted and the leaves are submerged into a bacterial suspension. The submerged plants are then placed in a vacuum desiccator for the infiltration procedure. Although both of these methods result in the rapid simultaneous inoculation of many plants, they still require observation of individual plants for a change in a visible defense-related phenotype.

We report here a 20-fold enrichment procedure for *Arabidopsis* mutants that cannot mount an HR that involves vacuum-infiltration of *Arabidopsis* seedlings growing on petri plates. The success of the method appears to depend on the fact that *P. s.* pv. *phaseolicola* NPS3121 is a nonhost pathogen of *Arabidopsis*, and therefore does not kill seedlings effectively by itself, and that NPS3121/*avrRpt2*, which elicits an HR in mature leaves, apparently elicits a systemic HR in seedlings that kills them.

Table 2. Linkage of *rps2-101C* to various genetic markers on chromosome IV[a]

marker[b]	No. of F_3 families[c]	Recombination events between *rps2-101C* and the marker in column 1	Percent recombination[d] (mean ± SD)
GA1	28	23	41.1 ± 6.6
AG	104	30	14.4 ± 2.4
19838	102	20	9.8 ± 2.1
PG11	104	2	1.0 ± 0.7
17340	102	5	2.5 ± 1.1
DHS1	96	29	15.1 ± 2.6

[a] Data in this table are derived from the La-*er* × *rps2-101C/rps2-101C* cross in Table 1.

[b] *GA1, AG, DHS1,* and PG11 are PCR-based markers on chromosome IV (Konieczny and Ausubel, in press), and 19838 and 17340 are RFLP markers on *Arabidopsis* chromosome IV described in AAtDB, an *Arabidopsis thaliana* database (Cherry *et al.* 1992).

[c] Because the RFLP markers are co-dominant, the number of chromosomes scored is twice the number of F_3 families.

[d] Percent recombination equals the number of recombination events observed divided by the total number of chromosomes scored.

Identification of an *Arabidopsis* resistance gene.

We used the seedling infiltration method described above as well as hand-inoculation to isolate two *Arabidopsis* mutants that could not mount an HR in response to *avrRpt2*. The following data suggest that the two rps2 mutants are specifically impaired in a function that involves signaling between *avrRpt2* and a single host gene, *RPS2*. First, the rps2 mutants displayed disease symptoms when infiltrated with *P. s.* pv. *maculicola* ES4326 or ES4326/*avrRpt2*. Second, ES4326/*avrRpt2* multiplied in the rps2 mutants at the same rate that *P. s.* pv. *maculicola* ES4326 multiplied in the wild type and in rps2 mutants' leaves. Third, these phenotypes of the rps2 mutants segregated in the F_2 and F_3 generations as expected for a single-gene trait when they were crossed to the wild-type parent. Fourth, the HRs normally elicited by ES4326/*avrB* or ES4326/*avrRpm1* were not impaired in the rps2 mutants.

Microscopic autofluorescence correlates with a visible HR.

To observe a macroscopic HR in *Arabidopsis*, it is necessary to infiltrate leaves with a relatively high dose of an avirulent bacterial strain (greater than 2×10^4 cells/cm^2). As shown in Figure 5, we found that avirulent strains that elicited a visible HR when infiltrated at a titer of 4×10^4 cells/cm^2 also elicited a microscopically observable autofluorescence (Schroder *et al.* 1992) and apparent cell death when infiltrated at a 10-fold lower titer. Importantly, rps2-101C did not respond to strains carrying *avrRpt2* at either the macroscopic or microscopic levels.

Semidominance at the *RPS2* locus.

The resistance response of heterozygous *RPS2*/*rps2* plants to *avrRpt2* was weaker than the resistance response in wild-type plants in two aspects. First, it took 4–8 hr longer and required at least two- to fivefold more ES4326/*avrRpt2* to elicit an HR in the heterozygous plants than in wild-type plants. Second, although an HR could be elicited in *RPS2*/*rps2* plants, the growth of ES4326/*avrRpt2* compared to *P. s.* pv. *maculicola* ES4326 was not limited 50- to 100-fold as it was in wild-type plants (Fig. 4). It is important to emphasize that the infiltration dose ($\sim 4 \times 10^3$ cells/cm^2) used to measure bacterial growth was too low to elicit a visible HR response, although a microscopic response could be detected. In fact, when infiltrated at this low dose, ES4326/*avrRpt2* often, but not always, elicited disease symptoms in *RPS2*/*rps2* plants that appeared over the course of 2–3 days. This latter result indicates that the heterozygous plants could be susceptible to a pathogen carrying *avrRpt2*, whereas homozygous *RPS2*/*RPS2* plants were never observed to be susceptible. Moreover, although HR-associated autofluorescence was observed in heterozygous plants infiltrated with the same dose of ES4326/*avrRpt2* that was used to carry out the growth experiments shown in Figure 4, fewer cells accumulated autofluorescence and it developed more slowly than in wild-type plants.

One explanation for the semidominance at the *RPS2* locus is a gene dosage effect, similar to that shown for a wheat resistance gene (Kerber and Dyck 1973). In wheat, as the resistance gene was diluted by crossing it from diploid into tetraploid and hexaploid lines, the effectiveness of the resistance was reduced in proportion to the number of R genes present. A second related possibility is that the products of the mutant *rps2* alleles interact with the product of the wild-type allele to reduce the activity of the resistance gene product in the heterozygote.

Response of the rps2 mutants to high pathogen doses.

An unexpected property of the rps2 mutants was that they exhibited different responses when infiltrated with high doses of *P. s.* pv. *maculicola* ES4326 or ES4326/*avrRpt2*. At very high doses ($>2 \times 10^5$ cells/cm^2) *P. s.* pv. *maculicola* ES4326 caused rapid tissue collapse in both wild-type and rps2 mutants, similar to the "normal sensitive necrosis" that has been described in other host-pathogen systems (Klement 1982). A high dose of ES4326/*avrRpt2* also caused rapid collapse in the wild-type plants, whereas no collapse was observed in the rps2 mutants. These results suggest that the rps2 mutants can still respond to the presence of *avrRpt2* in *P. s.* pv. *maculicola* ES4326. One explanation for this result is that the rps2 mutants are leaky and are still capable of recognizing an *avrRpt2*-generated signal. Another possibility is that there is a second receptor that recognizes the *avrRpt2*-generated signal. A third possibility is that *P. s.* pv. *maculicola* ES4326 itself produces an elicitor that causes plant necrosis at high doses. However, the *avrRpt2* gene product is involved in converting this elicitor to a different form that is a more potent elicitor of the HR that is recognized by the *RPS2* product. Thus, rps2-101C and rps2-102C cannot respond to ES4326/*avrRpt2* because most or all of the *P. s.* pv. *maculicola* ES4326–specific elicitor is converted into the form recognized by the *RPS2* product. A final possibility is that the *avrRpt2* product leads to the synthesis of more than one elicitor that interacts with different plant receptors.

The *RPS2* locus.

In addition to the two rps2 mutants described in this paper, (Kunkel *et al.*, *in press*) isolated four *Arabidopsis* ecotype Columbia mutants that give disease symptoms instead of a resistance response when dipped into a *P. s.* pv. *tomato* strain DC3000/*avrRpt2* suspension containing Silwet L77. As shown in Table 1, when one of these mutants, *rps2-201C*, was crossed with *rps2-101C*, among 254 F_2 progeny tested, none gave an HR when infiltrated with ES4326/*avrRpt2*. A similar result was obtained when the two rps2 mutants isolated in our laboratory were crossed to each other (Table 1). Moreover, both *rps2-101C* and *rps2-201C* map at approximately the same location on chromosome IV (Fig. 6 and Kunkel *et al.*, *in press*). Although these data strongly suggest that the rps2 mutants isolated to date as allelic, additional molecular characterization is required to determine the detailed structure of the *Arabidopsis RPS2* locus.

MATERIALS AND METHODS

Bacterial strains and *avr* genes.

P. s. pv. *maculicola* strain ES4326 (Dong *et al.* 1991; Whalen *et al.* 1991) and *P. s.* pv. *phaseolicola* NPS3121 (Lindgren *et al.* 1986) have been described. The cosmid

cloning vector pLAFR3 (Swanson *et al.* 1988) and the avirulence genes *avrRpt2* (on plasmid pLH12) (Whalen *et al.* 1991), *avrRpm1* (on plasmid K48) (Debener *et al.* 1991), and *avrB* (on plasmid pPSG0002) (Staskawicz *et al.* 1987) have been described. *P. syringae* strains were grown at 28° C in King's medium B (King *et al.* 1954), and *Escherichia coli* strains were grown at 37° C in LB medium (Sambrook *et al.* 1989). Culture media were supplemented with 50 μg/ml of streptomycin, 50 μg/ml of rifampicin, or 15 μg/ml of tetracycline as required. Conjugation of plasmids pLAFR3, pLH12, K48, and pPSG0002 was carried out by triparental mating using the helper plasmid pRK2013 as described by Ditta *et al.* (1980).

Growth of *Arabidopsis* plants and hand-infiltration of *Pseudomonas* strains.

Arabidopsis seeds were germinated and grown in Metro-Mix 200 (W. R. Grace, Inc.) for 2 wk in a climate-controlled greenhouse (22° C) with supplemental fluorescent lighting (16-hr photo period) and then transferred to a growth chamber at 22° C with a photo period of 12 hr and a light intensity of 100 $\mu E \cdot s^{-1} \cdot m^{-2}$. Plants with well-expanded rosettes (4- to 6-wk old) were infiltrated with *Pseudomonas* strains that had been grown overnight in King's medium B and resuspended in 10 mM $MgCl_2$. The number of bacterial cells used for inoculation was determined by using the observation that an OD_{600} of 0.002 is equivalent to $\sim 4 \times 10^3$ cells/cm^2 of leaf area. An appropriate dilution (in 10 mM $MgCl_2$) was infiltrated into leaves using a 1-ml syringe without a needle to force about 10 ml of a bacterial suspension through the stomatal openings on the bottom side of the leaves (Swanson *et al.* 1988).

Growth and vacuum-infiltration of *Arabidopsis* seedlings with *Pseudomonas* strains

Approximately 200 sterilized seeds were spread on 9-cm agar plates containing vitamin supplemented MS medium (Murashige and Skoog 1962) and cold-treated at 4° C for 2–3 days. Seedlings were incubated in a tissue culture growth room at 22° C under fluorescent lights with an intensity of 50–100 $\mu E \cdot s^{-1} \cdot m^{-2}$. Two-week-old seedlings were submerged in a bacterial suspension in 10 mM $MgCl_2$ and placed in a vacuum desiccator. Vacuum was applied until gas bubbles escaped from the leaves and cotyledons. When the vacuum was released, bacteria were sucked through stomatal openings (Hildebrand *et al.* 1988). Seedlings were washed with sterile water three times and air-dried in a tissue culture hood for 2 hr. Infiltrated seedlings were incubated for 5 days at 22° C under constant fluorescent light with an intensity of 50–100 $\mu E \cdot s^{-1} \cdot m^{-2}$.

Bacterial growth in *Arabidopsis* leaves.

Bacterial growth in leaves was measured by determining the average of the logarithm of the number of viable bacteria in six leaf disks at each time point. Leaf disks (0.28 cm^2), punched outside of the initial infiltration site, were ground in 10 mM $MgCl_2$ in Eppendorf tubes using a plastic pestle. Appropriate dilutions were plated on King's medium B supplemented with 50 μg/ml of streptomycin.

Microscopy.

One day after infiltration with *P. s.* pv. *maculicola*, *Arabidopsis* leaves were removed from the plants and fixed in FAA (2% formaldehyde, 5% acetic acid, 40% ethanol) for 15 min. The fixed leaf tissues were soaked in 50% ethanol for 20 min and then incubated in 95% ethanol overnight to remove chlorophyll. Palisade parenchyma cells were observed from the top side of the leaves under a fluorescent microscope (Ex = 460, Em > 478) (Zeiss universal M light microscope).

Genetic and RFLP analysis.

Genetic crosses were performed by dissecting unopened flower buds and brushing donor pollen on the exposed carpels. Isolation of *Arabidopsis* genomic DNA, restriction endonuclease digestion, and DNA blot analysis were carried out as described (Ausubel *et al.* 1993). DNA was isolated from pooled F_3 families and purified by CsCl equilibrium gradient centrifugation. PCR-based mapping was carried out as described (Konieczny and Ausubel, *in press*). Briefly, primers were designed from previously sequenced and mapped genes and used to amplify DNA isolated from pooled F_3 families. The PCR products were than digested with a restriction enzyme, which revealed polymorphic bands between the Columbia and Landsberg *erecta* ecotypes.

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