A Single Locus Leads to Resistance of *Arabidopsis thaliana* to Bacterial Wilt Caused by *Ralstonia solanacearum* Through a Hypersensitive-like Response

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ABSTRACT

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Strains of *Ralstonia solanacearum* have been shown to cause bacterial wilt in some, but not all, ecotypes of *Arabidopsis thaliana*. We demonstrate here that after inoculation of the leaves of resistant ecotype S96 with *R. solanacearum* strain Ps95 necrosis around the inoculation site rapidly appeared and no further symptoms developed in the plant. Leaves of susceptible ecotype N913 completely wilted 7 days after inoculation with Ps95, and symptoms spread systemically throughout the whole plant within 2 weeks

after inoculation. These results suggest that the resistance of *Arabidopsis* S96 to *R. solanacearum* is due to a response similar to the hypersensitive response (HR) observed in other plant diseases. Northern blot analysis of the expression of defense-related genes, known to be differentially induced during the HR in *Arabidopsis*, indicated that pathogenesis-related protein *PR-1*, glutathione S-transferase (*GST1*), and Cu,Zn superoxide dismutase (*SOD*) mRNAs increased significantly in S96 leaves between 3 to 12 h after infiltration with Ps95. The induction of these genes in susceptible ecotype N913 by Ps95 was clearly delayed. Genetic analysis of crosses between resistant ecotype S96 and susceptible ecotype N913 indicated that resistance to Ps95 is due to a single dominant locus.

Ralstonia solanacearum, a bacterial plant pathogen, causes a serious wilt disease and severe losses to many agricultural crops throughout the world (14). To develop useful strategies to control this disease, it is very important to investigate mechanisms of plant resistance. *Arabidopsis thaliana* (L.) Heynh. has been successfully used as a model host plant for several bacterial pathogens including *R. solanacearum* (10,18,27,34). A variety of disease responses were observed among different *Arabidopsis* ecotypes 2 weeks after inoculation with different strains of *R. solanacearum*. Both susceptible and resistant ecotypes of *Arabidopsis* were identified (34). The susceptible ecotypes develop systemic wilt, whereas the resistant ecotypes remain asymptomatic after infection. The degree of pathogenicity of different strains of *R. solanacearum* was correlated with the rapid distribution of the bacterium throughout the shoot of susceptible plants. This suggests that the ability of resistant *Arabidopsis* ecotypes to limit the multiplication and movement of *R. solanacearum* through the plant is a major factor in their disease resistance (34).

The restriction of various pathogens to the inoculation site in resistant interactions has been widely reported (3,7,12,23,30). A rapid necrotic response, called the hypersensitive response (HR), often results from gene-for-gene recognition (16), although the molecular mechanisms of HR elicitation are still unclear. Since resistance to bacterial wilt in different tomato varieties has been demonstrated genetically to be controlled by quantitative trait loci (1,9,26,31,32), the basis of the resistance of *Arabidopsis* to *R. solanacearum* provides a useful model system.

In this study, we investigated the mechanism of resistance of *Arabidopsis* ecotype S96 to *R. solanacearum*. Our results demonstrate that rapid cell death, similar to a HR, occurred at the inoculation site and was strongly correlated with the resistance. A ne-

crotic zone around the inoculation or infiltration site was observed only in resistant plants, whereas systemic infection developed in susceptible plants.

This hypersensitive-like response is very similar to that observed in *Arabidopsis* plants infected with other bacterial pathogens, in which bacterial growth is either reduced or directly limited to the site of inoculation (18,27). Single resistance genes are involved in the resistance of *Arabidopsis* to *Pseudomonas syringae* (*RPS2* and *RPM1*) and *Xanthomonas campestris* (*RXC*) (3,6,12,18,23,27). Here, we analyze the genetic basis of the resistance in *Arabidopsis* to *R. solanacearum*. As in other HR systems, a single dominant locus, identified in resistant ecotype S96, is genetically linked for this hypersensitive-like response and resistance to *R. solanacearum* strain Ps95. This finding suggests that the *Arabidopsis*-*R. solanacearum* interaction could be an excellent model system that could lead to the cloning of additional resistance genes in the future.

MATERIALS AND METHODS

Plant growth conditions. Seeds of *Arabidopsis* ecotypes N913 and S96 were obtained from the *Arabidopsis* Biological Resource Center, The Ohio State University, Columbus. Seeds were sterilized and placed on agar plates containing $1/2\times$ Murashige & Skoog medium (24), kept at 4°C for 2 days and then germinated in growth chambers under long-day conditions (16-h light/8-h dark) for 10 days before being transplanted to the greenhouse. The light intensity of the growth chambers was $150\ \mu\text{E m}^{-2}\text{ s}^{-1}$. Seedlings were transplanted to soil and grown in greenhouses for different times before bacterial inoculation. The greenhouses were maintained at 22°C with 16 h of light for long-day conditions.

Bacterial inoculation. The Taiwanese strains of *R. solanacearum*, Rd15 (from radishes) and Ps95 (from tomatoes), used in this study are both race 1 and virulent to sweet pepper, eggplant, and tobacco (34). Bacteria were grown on a tetrazolium chloride selective agar medium (17) at 28°C for 4 days before inoculation. Two methods were used to introduce bacteria into the leaves of

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Arabidopsis. In the first method, 17-day-old plants were inoculated by puncturing the surface of the rosette or cauline leaves with a needle dipped into a bacterial colony. The number of bacteria used for each needle puncture was determined by a serial dilution plating and was approximately 10^8 CFU per plant. In the second method, bacterial suspensions (10^8 CFU/ml) were infiltrated with a syringe into a 1- to 2-cm² area of the leaf through a hole made by a needle puncture. The infected plants were grown under long-day conditions (16-h light/8-h dark) at 25°C in a growth chamber until symptoms appeared. Inoculations using each method were done three times using at least five plants of each ecotype for each strain in each experiment.

RNA gel blot analysis. For RNA isolation, 100 mg of fresh leaf tissue was homogenized with 1 ml of homogenization solution (ULTRASPEC RNA isolation system; BIOTECX Company, Hous-

ton). The homogenate was stored at 4°C for 5 min and 0.2 ml of chloroform per 1 ml of homogenate was added. The samples were covered tightly, shaken vigorously for 15 s, and kept on ice at 4°C for 5 min. After centrifugation for 15 min at $12,000 \times g$ (4°C), an equal volume of isopropanol was added to the aqueous phase and samples were stored for 10 min at 4°C. RNA was precipitated by centrifugation for 10 min at $12,000 \times g$ (4°C), washed twice with 75% ethanol, and centrifuged for 5 min at $7,500 \times g$ (4°C). RNA was dried under vacuum and resuspended in diethylpyrocarbonate-treated water.

For northern hybridizations, 5 µg of total RNA was electrophoresed in formaldehyde agarose gels (2) and transferred to Hybond N+ membranes (Amersham International, Buckinghamshire, United Kingdom). The membranes were prehybridized for 30 min, hybridized with a ³²P-labeled DNA probes overnight in the same solu-

TABLE 1. Oligonucleotide primers used for the polymerase chain reaction (PCR) amplification of DNA probes

Gene	Sense primer	Antisense primer	PCR product (bp) ^a	Reference
<i>PR-1</i>	GTAGGTGCTCTGTTCCTCCC	CACATAATCCACGAGGATC	420	33
<i>GSTI</i>	ATGGCAGGAATCAAAGTTTCG	CAAGACTCATTATCGAAGATTAC	1,016	29
<i>Cu,Zn SOD</i>	GCGAAAGGAGTTGCAGTTTGAACA	ACACGGCCGCTGCGTTTCCAGTAG	1,100	15

^a Genomic DNA as a template for PCR reaction.

Fig. 1. Symptom development in leaves of *Arabidopsis* plants N913 and S96 after puncture inoculation or infiltration with *Ralstonia solanacearum* strain Ps95. **A**, Leaf from a N913 plant 10 days after inoculation with Ps95. The leaf was wilted entirely and the plant was dead at 14 days after inoculation. **B**, Close-up of the inoculation site from **A**. **C**, Leaf from a N913 plant 5 days after infiltration with Ps95. The leaf was wilted entirely. **D**, Leaf from a N913 plant 10 days after infiltration with Ps95. The leaf was completely dried and the plant was dead. **E**, Leaf from a S96 plant 2 days after inoculation with Ps95. Necrosis of cells around the inoculation site was clearly observed. **F**, Close-up of the inoculation site from **E**. **G**, Leaf from a S96 plant 1 day after infiltration with Ps95. The infiltrated area of the leaf began to show yellowing. **H**, Leaf from a S96 plant 2 days after infiltration with Ps95. The infiltrated area of the leaf turned brownish. **I**, Leaf from a S96 plant 4 days after infiltration with Ps95. The infiltrated area was necrotic, whereas other parts of the leaf remained green. **J**, Same as the leaf in **I**. The necrosis was confined to the infiltrated area. **K**, Leaf from a control S96 plant 7 days after inoculation with water. Cells around the inoculated site (the hole) have no sign of any damage or necrosis. **L**, Close-up of the inoculation site from **K**, which is distinct from that observed in **E**.

tion (0.25 M Na_2HPO_4 , pH 7.2, and 7% sodium dodecyl sulfate [SDS]), and then washed twice each in solution 1 (20 mM Na_2HPO_4 , pH 7.2, and 5% SDS) and solution 2 (20 mM Na_2HPO_4 , pH 7.2, and 1% SDS) for 30 min per wash. The blots were then air-dried, covered with plastic wrap, and autoradiographed. The DNA probes specific for defense genes *PR-1* (420 bp), *GST1* (1,016 bp), and *Cu,Zn SOD* (1,100 bp) were amplified through polymerase chain reactions (PCRs). About 50 ng of genomic DNA was used as a template for 35 cycles of PCR amplification. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μM of each deoxynucleoside triphosphate, and 0.1 μM of each oligonucleotide primer in a volume of 50 μl . The denaturing step was at 94°C for 1 min, the annealing step was at 55°C for 2 min, and the extension step was at 72°C for 2 min. The primers used for PCR amplification are listed in Table 1.

Genetic analysis. To analyze the genetic basis of resistance, plants of ecotype N913 (susceptible to Ps95) were crossed with plants of ecotype S96 (resistant to Ps95). F_1 plants were self-pollinated, and the F_2 progeny were inoculated with Ps95 by infiltration of leaves at different developmental stages. The resistant and susceptible plants were counted. To further confirm the result, backcrosses were also made ($F_1 \times \text{S96}$ and $F_1 \times \text{N913}$) and the progeny were tested for resistance to Ps95.

RESULTS

Resistance of *Arabidopsis* to *R. solanacearum* is associated with a hypersensitive-like response. Strains of *R. solanacearum* caused bacterial wilt symptoms on *Arabidopsis* plants similar to those observed in solanaceous crops, i.e., leaf wilting, discoloration, leaf drop, stunting, and finally permanent wilting and death (34). When bacteria were inoculated directly into the inflorescence stem of susceptible plants, as reported previously, it was difficult to observe any disease response at the infection site. In this study, we inoculated bacteria into leaves by needle puncture or infiltration and clear differences between susceptible and resistant ecotypes were observed. For example, Rd15 caused severe symptoms in both ecotypes N913 and S96, whereas Ps95 caused symptoms only in ecotype N913 and not in S96. In all susceptible cases, symptom development was slightly slower than previously reported for inflorescence inoculation. With Rd15, symp-

toms appeared after 1 to 3 days in the inoculated leaves of both S96 and N913 plants and the leaves were completely wilted by 7 days after infection. Symptoms rapidly spread to other parts of the plant, and plants were completely wilted by 10 days after infection. This is slightly later than with inflorescence inoculation, in which the plants completely wilted by 7 days after infection (34).

Leaves of N913 plants inoculated with Ps95 showed symptoms after 3 to 5 days and were completely wilted by 10 days after infection (Fig. 1A and B). Symptoms spread slower than in plants

Fig. 2. Northern blot analysis showing the induction of *PR-1*, *GST1*, and *Cu,Zn SOD* mRNAs in leaves of *Arabidopsis* S96 and N913 plants after infiltration with *Ralstonia solanacearum* strain Ps95. Total RNA was extracted from the infiltrated area of leaves from three different S96 or N913 plants at different times after infiltration (1, 3, 6, 12, 24, and 48 h) with Ps95. About 5 μg of mRNA was used for each sample. Time in hours after infiltration is indicated above the blots. C indicates the noninfiltrated control plant. 18S rRNA served as an internal control. Each experiment was repeated twice with similar results.

Fig. 3. Induction of *PR-1*, *GST1*, and *Cu,Zn SOD* in *Arabidopsis* ecotypes S96 and N913 at different times after infiltration with *Ralstonia solanacearum* strain Ps95. The strength of the signals in northern blots (Fig. 2) were measured with a PhosphorImager (Fuji Photo Film Co., Tokyo). The values of the hybridization signal obtained for *PR-1*, *GST1*, and *Cu,Zn SOD* were normalized to the values obtained for the 18S rRNA control probe for each time point. The normalized value for noninoculated control at time 0 was set equal to 1, and the resulting relative values for each time point were plotted. Data points are the means of two independent experiments that showed similar results.

infected with Rd15, and plants were completely wilted by 14 days after infection. Similar symptom development was observed when bacteria were introduced into the leaves by infiltration (Fig. 1C and D).

In the resistant interaction, S96 inoculated with Ps95, wilt symptoms were not observed in any part of the plants by 14 days after inoculation. Interestingly, after inoculation of the bacteria by direct needle puncture into the leaves, the tissue surrounding the inoculation sites became necrotic as early as 1 day after inoculation. These cells completely died 2 to 3 days after inoculation and no further necrosis developed (Fig. 1E and F). This rapid necrotic response prevented further disease development and appeared to be a HR. Similar results were observed when bacteria were infiltrated into the leaves. The infiltrated area became chlorotic within 1 day (Fig. 1G) and then turned brownish (Fig. 1H) and necrotic, whereas other parts of the leaf remained green (Fig. 1I and J). The visible necrosis clearly corresponded to the infiltrated zone (Fig. 1J). Control leaves inoculated or infiltrated with water remained green and symptomless (Fig. 1K and L), indicating that the necrosis was caused by the bacterial infection and not by wounding.

PR-1, GST1, and Cu,Zn SOD mRNAs were differentially induced in resistant and susceptible *Arabidopsis* leaves after infiltration with Ps95. To investigate if the expression of defense-related or pathogenesis-related genes in *Arabidopsis* could be induced by infection with *R. solanacearum*, mRNA from leaves of *Arabidopsis* plants S96 and N913 after infiltration with Ps95 was isolated at various times and assayed using northern hybridization. As shown in Figures 2 and 3, the level of *PR-1* transcripts increased significantly in S96 12 h after inoculation (about 20-fold more than in noninoculated control plants). The amount of *PR-1* mRNA remained at the same level for 24 h after infiltration and decreased to a basal level at 48 h. In contrast to S96, *PR-1* transcripts in N913 only increased about eightfold over controls by 24 h after infiltration with Ps95. The amount of *PR-1* mRNA declined to half at 48 h after infiltration. This result clearly indicates that *PR-1* mRNA was induced earlier and more abundantly in resistant plants than in susceptible plants. In leaves infiltrated with water as controls, the amount of *PR-1* mRNA was detected at a basal level similar to that of untreated leaves. This indicated that the induction of mRNA was caused by the bacterial infection and not by wounding.

The induction of the *GST1* gene in infiltrated plants was similar to that observed for *PR-1* as shown in Figures 2 and 3. In S96 plants infiltrated with Ps95, *GST1* transcripts peaked at 6 h after infiltration, reaching a level three- to fourfold higher than in non-inoculated controls. The amount of *GST1* mRNA remained at the same level until 12 h and then decreased to a basal level at 48 h. In the susceptible interactions, the induction of *GST1* transcripts was delayed in N913 plants at 12 h after infiltration with Ps95. The *GST1* mRNA decreased gradually to a basal level by 48 h after infiltration. In control plants, the *GST1* transcripts were not induced by infiltration with water as shown in Figure 2.

Cu,Zn SOD was also differentially induced in resistant and susceptible ecotypes after infiltration with Ps95. As shown in Figures 2 and 3, Cu,Zn SOD transcripts were induced in S96 plants after only 3 h and peaked at 12 h; maximum levels were two- to threefold more than in control plants. The amount of Cu,Zn SOD mRNA decreased between 24 and 48 h after infiltration. In the susceptible

interaction, the induction of Cu,Zn SOD transcripts was delayed in N913 plants at 12 h after infiltration with Ps95. The Cu,Zn SOD mRNA decreased to a basal level at 48 h.

Genetic analysis of resistance to *R. solanacearum* Ps95 in ecotype S96. Since different ecotypes of *Arabidopsis* exhibit differential responses to Ps95 (34), genetic analysis of the nature of this resistance and the identification of any resistance genes are important. Crosses between resistant ecotype S96 and susceptible ecotype N913 were made, and F₂ segregating populations were obtained. As shown in Table 2, after infiltration of Ps95 into the leaves of 101 30-day-old N913 × S96 F₂ plants, 76 of them showed a resistant response by only producing necrosis at the infiltration site (Fig. 4, right). The remaining 25 F₂ plants showed a susceptible phenotype and developed typical systemic wilt symptoms by 7 days after infiltration (Fig. 4, left). This 3:1 ratio of resistant to susceptible plants suggests that a dominant resistance locus is present in ecotype S96 and is absent in ecotype N913. To confirm this result, younger F₂ plants were infiltrated with Ps95 and symptom development was recorded. As shown in Table 2, a clear 3:1 ratio of resistant to susceptible plants was observed again in both 10- and 20-day-old F₂ populations. When the data from these three independent experiments were combined, a more significant segregation ratio for confirming linkage of a hypersensitive-like response to wilt symptoms was obtained (265:86, $\chi^2_{3:1} = 0.0443$, $P > 0.5$).

To confirm single-locus inheritance, further genetic analysis was performed. Whereas 16 progeny from a first backcross, F₁ (N913 × S96) × S96, were all resistant, 7 progeny from a second backcross, F₁ (N913 × S96) × N913, segregated into three resistant and four susceptible progeny. All of these backcross progeny plants were then self-fertilized and the phenotypes of their progenies were analyzed. From the first backcross, 7 out of the above 16 individuals gave a 3:1 ratio of resistant to susceptible progeny and 9 out of 16 gave progeny that were all resistant (7:9, $\chi^2_{1:1} = 0.25$, $P > 0.5$). From the second backcross, three resistant individuals gave a 3:1 ratio of resistant to susceptible progeny and four susceptible individuals gave progeny that were all susceptible (3:4, $\chi^2_{1:1} = 0.14$, $P > 0.5$). This result strongly supports the conclusion that resistance is due to a single dominant gene and is not influenced by plant age. Ecotype N913 clearly lacks resistance to any of the tested strains of *R. solanacearum* and, therefore, is highly susceptible to infection by Ps95.

DISCUSSION

In our previous study, *Arabidopsis* was demonstrated to be a host plant for *R. solanacearum* and both resistant and susceptible ecotypes were identified (34). Bacteria were unable to multiply and spread throughout the resistant plants (34). Using the leaf-inoculation procedures described here, development of wilting symptoms was identical to that observed after inoculation of the inflorescence stem. Leaf inoculation provides an easier and more efficient way to study the interactions between *Arabidopsis* and *R. solanacearum*.

In resistant plants, rapid necrosis of cells adjacent to the infection site in the leaves was correlated with reduced movement of the bacteria away from the inoculation site. This observation supports the notion that a mechanism limiting the spread of bacteria exists in resistant plants (34) and it should function in both stem and leaf the same way. The necrosis of cells caused by this mech-

TABLE 2. Segregation analysis of *Ralstonia solanacearum* strain Ps95 resistance in F₂ progeny from crosses between *Arabidopsis* ecotypes N913 (susceptible) and S96 (resistant)

Cross	Inoculated stage ^a	Resistant ^b	Susceptible ^c	Expected ratio (R:S)	χ^2	P
N913 × S96	10	99	32	3:1	0.023	0.88
N913 × S96	20	90	29	3:1	0.0252	0.87
N913 × S96	30	76	25	3:1	0.0033	0.96

^a Indicates the days after germination when plants were inoculated with bacteria.

^b Resistant (R) = Ten days after infiltration with strain Ps95, only cell death in the infiltrated area of the leaf was observed; the plant was completely healthy.

^c Susceptible (S) = Ten days after infiltration with strain Ps95, the infiltrated leaf was wilted entirely and the plant showed severe symptoms.

anism is similar to the HR reported in resistant responses of many plants including the interactions between *Arabidopsis* and *P. syringae* (3,12,23), in which the inoculation of certain *P. syringae* strains on resistant *Arabidopsis* ecotypes caused cell death and limitation of bacterial growth and spread. In addition, certain strains of *R. solanacearum* have been reported to be able to induce a HR in tobacco leaves after infiltration (4,8).

It is well known that certain defense genes are induced during the HR caused by various pathogens and may be involved the inhibition of pathogen growth (5,8,13,19,20–22,25,28,29). In general, these defense genes are induced in both incompatible and compatible reactions, but their expression is usually delayed or weaker in the susceptible response (11,33). In this study, we show that the expression of three defense genes, *PR-1*, *GST1*, and Cu,Zn *SOD*, is induced by infection with Ps95 in both resistant (S96) and susceptible (N913) *Arabidopsis* ecotypes. However, the induction of these three genes is earlier and stronger in S96 than in N913 plants. Their expression pattern provides additional evidence that the resistance of S96 to Ps95 is an elicited response similar to the HR.

Arabidopsis resistance genes such as *RPS2* and *RPM1* that confer resistance to *P. syringae* have been identified and cloned (3,12,23). Like many other genes involved in a HR, *RPS2* and *RPM1* are both inherited as single dominant loci. Since the HR-like resistance observed in ecotype S96 also appears to be inherited as a single dominant trait, we propose that infection by Ps95 triggers a mechanism similar to a HR, which is conditioned by the resistance locus identified here. This mechanism causes rapid induction of defense genes and necrosis at the inoculation site, which prevents the further spread of the bacteria in the resistant plants. In susceptible plants (N913), the absence of this locus results in the delay of defense gene expression. The pathogen then multiplies rapidly and spreads throughout the plant, resulting in death. To confirm this observation and investigate the resistance mechanism, efforts are underway to analyze more crosses made from various combinations between resistant and susceptible ecotypes and to test the allelism of the resistance genes identified in different ecotypes. Further mapping and cloning of the resistance genes identified in S96 are also in progress.

Fig. 4. Resistant and susceptible F_2 progeny from the cross between *Arabidopsis* ecotypes N913 and S96 after inoculation with *Ralstonia solanacearum* strain Ps95. No symptoms were observed in the resistant plant (right), whereas leaves showed severe wilting in the susceptible plant (left) at 7 days after inoculation.

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