The Plant Cell, Vol. 10, 1251–1266, August 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

The Tomato *Cf-9* Disease Resistance Gene Functions in Tobacco and Potato to Confer Responsiveness to the Fungal Avirulence Gene Product Avr9

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The *Cf-9* gene encodes an extracytoplasmic leucine-rich repeat protein that confers resistance in tomato to races of the fungus *Cladosporium fulvum* that express the corresponding avirulence gene *Avr9.* We investigated whether the genomic *Cf-9* gene functions in potato and tobacco. Transgenic tobacco and potato plants carrying *Cf-9* exhibit a rapid hypersensitive cell death response (HR) to Avr9 peptide injection. Cf9 tobacco plants were reciprocally crossed to Avr9-producing tobacco. A developmentally regulated seedling lethal phenotype occurred in F_1 progeny when Cf9 was used as the male parent and Avr9 as the female parent. However, when Cf9 was inherited in the maternal tissue and a heterozygous Avr9 plant was used as the pollen donor, a much earlier reaction was caused, leading to no germination of any F_1 seed. Detailed analysis of the Avr9-induced responses in Cf9 tobacco leaves revealed that (1) most mesophyll cells died within 3 hr (compared with 12 to 16 hr in tomato); (2) the macroscopic HR was visible at an Avr9 titer five times lower than that which caused visible symptoms in tomato; (3) the HR invariably extended into noninjected panels of the tobacco leaf; (4) no HR occurred in leaves of young tobacco plants; (5) in older plants, the HR was dramatically enhanced by sequential Avr9 challenges; and (6) coexpression of a salicylate hydroxylase transgene (*nahG*) from *Pseudomonas putida* reduced the severity of the macroscopic leaf HR and also restored germination to Cf9 × *35S:Avr9* F_1 seedlings. Simultaneous introduction of *Cf-9* homologs (*Hcr9-9* genes A and B or D) along with the native *Cf-9* gene did not alter the responses that were specifically induced by Avr9. Various ways to use the *Cf-9–Avr9* gene combination to engineer broad-spectrum disease resistance in several solanaceous species are discussed.

INTRODUCTION

Resistance (*R*) genes and their products play a pivotal role in the plant's surveillance system that recognizes potentially pathogenic microbes and then activates the appropriate defense responses (Keen, 1990). For *R* gene–dependent resistance to occur, as predicted by Flor's gene-for-gene hypothesis (Flor, 1971), the pathogen must express the complementary avirulence (*Avr*) gene that encodes, either directly or indirectly, a product recognizable by the plant. If either the *R* or *Avr* gene is absent or nonfunctional, disease will ensue.

The isolation of several *R* genes involved in diverse plant–pathogen interactions, conferring resistance to fungi, bacteria, viruses, and nematodes (Bent, 1996; Baker et al., 1997; Hammond-Kosack and Jones, 1997), has revealed four related but distinct classes of predicted R proteins that confer resistance conforming to Flor's gene-for-gene hypothesis. These *R* genes from tomato, tobacco, rice, flax, and Arabidopsis encode proteins that share one or more similar motifs: leucine-rich repeat (LRR) and Toll/interleukin/resistance (TIR) regions (implicated in protein–protein interactions), nucleotide binding sites (NBSs), and protein kinase domains (implicated in signaling). The predicted R protein structures strongly suggest that they function as receptors in a signal transduction pathway that coordinates the activation of the multifaceted plant defense response.

Wild relatives or closely related species of cultivated plants are useful sources of additional *R* genes for plant breeding programs. From such breeding work, it is known that certain *R* genes function when transferred to a sexually compatible species. The isolation of *R* genes now makes possible their introduction, via transformation, into sexually incompatible plant taxa. However, the extent to which the transferred *R* genes function in other plant taxonomic groups is unpredictable. Similar motifs in different R proteins from species as distantly related as rice, Arabidopsis, tomato, tobacco, and flax suggest that mechanisms to combat attacks from microbes may be conserved in different plant families.

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The different classes of R proteins probably regulate distinct recognition and signaling pathways that then converge to activate the common array of defense mechanisms. By using mutational analysis, several additional loci required for disease resistance have been defined as required for full expression of *R–Avr*—mediated disease resistance (Hammond-Kosack and Jones, 1996; Baker et al., 1997). One of these additional loci codes for a protein with motifs in common with R proteins; tomato Prf required for *Pto* function contains both NBS and LRR domains (Salmeron et al., 1996). In contrast, the Arabidopsis *NDR1* gene required for *Rps2* and *Rpm1* function encodes a putatively membrane-associated product without homology to known *R* gene products (Century et al., 1997). The *Eds1* gene is required for the function of several but not all Arabidopsis *R* genes (Parker et al., 1996). In addition, all *R* genes characterized so far require salicylic acid (SA) levels to be elevated to achieve complete avirulent pathogen containment (Gaffney et al., 1993; Delaney et al., 1994). This suggests that *R* and *Avr* gene–dependent defense operates through a highly integrated network of signal events and contains various positive and negative checkpoints for both fine tuning and cross-talk. The combined data arising from the isolation of *R* genes and genes required for *R* gene function also suggest that the evolution of specificity in plant–pathogen interactions can occur at several distinct points within the network. These mechanisms appear to be conserved among closely related species. For example, the tomato *Pto* and *Fen* genes have been shown to function in *Nicotiana* spp, and the tobacco *N* gene is active in tomato (Rommens et al., 1995; Thilmony et al., 1995; Whitham et al., 1996).

In this study, we evaluate whether the tomato *Cf-9* gene functions in tobacco and potato. The tomato *Cf-9* gene confers resistance to races of *Cladosporium fulvum* that express the complementary avirulence gene *Avr9* (De Wit et al., 1985). Although this leaf mold fungus is solely a pathogen of tomato (Bond, 1938), the extracellular mode of biotrophic hyphal colonization allows the recovery of the primary products of various *Avr* genes from intercellular washing fluids (IFs) isolated from leaves supporting heavy fungal sporulation (De Wit and Spikman, 1982). This subsequently led to the isolation of two *C. fulvum* avirulence genes, *Avr9* and *Avr4* (Van Kan et al., 1991; Joosten et al., 1994). The products of both *Avr* genes are secreted from fungal cells and processed by both fungal and plant proteases to produce peptides of 28 amino acids (Avr9) and 106 amino acids (Avr4). Avr9 and Avr4 are cysteine-rich basic peptides that elicit a rapid macroscopic gray necrotic response when injected into the airspaces of healthy cotyledons or leaves of tomato cultivars carrying *Cf-9* and *Cf-4*, respectively (Van Kan et al., 1991; Joosten et al., 1994). Therefore, although *C. fulvum* cannot colonize either tobacco or potato, an Avr9 elicitor peptide injection assay can be used to determine whether *Cf-9* functions in plant species that are non-hosts to *C. fulvum.* In addition, transgenic tobacco plants were available that harbor an *Avr9* transgene under the control of the cauliflower mosaic virus 35S promoter (*35S:Avr9* T-DNA) and that secrete high levels of Avr9 peptide into the plant apoplast (Hammond-Kosack et al., 1994a). Therefore, it was possible to test whether the developmentally regulated seedling lethal phenotype evident in (Cf9 × *35S:Avr9*) F_1 tomato seedlings occurred in another plant species. This analysis revealed an unexpected "no seed germination" phenotype when *Cf-9* was in the female parent, which could be alleviated by coexpression of the *Pseudomonas putida nahG* salicylate hydroxylase gene.

RESULTS

Introduction of *Cf-9* Genomic Sequences into Tomato, Tobacco, and Potato

Several genomic *Cf-9* transformants were selected to ascertain whether the presence of the linked *Cf-9* homologs was required for Cf-9 function in heterologous plant species. This possibility existed because in the original transposon-tagging experiment used to isolate *Cf-9*, *R* gene function was always assessed in the presence of all of the other *Hcr9-9* genes (Jones et al., 1994; Parniske et al., 1997).

Two characterized cosmid clones carrying *Cf-9* (Thomas et al., 1995) were selected for plant transformation. Cosmid 2/9-34 carries an intact copy of the *Cf-9* gene as well as intact copies of two *Cf-9* gene homologs, *Hcr9-9A* and *Hcr9-9B* (Parniske et al., 1997), located 5′ of the *Cf-9* promoter region, as shown in Figure 1. The second cosmid clone, 2/9-110, carries an intact copy of the *Cf-9* gene and an intact copy of the *Hcr9-9D* homolog located 3′ of the *Cf-9* gene. *Cf-9* and each *Hcr9-9* gene are transcribed in the same direction, and the two clones contain no other gene sequences (Parniske et al., 1997). The total length of genomic DNA covered by cosmid 2/9-34 and cosmid 2/9-110 is 18.5 and 16.5 kb, respectively. A PstI fragment containing the *Cf-9* gene was subcloned from cosmid 2/9-34 into a binary vector SLJ7292 to create the construct SLJ8808. This clone contained the *Cf-9* gene in conjunction with 4581 bp of sequence 5′ from the ATG and 468 bp of sequence 3′ from the termination codon. Additional details regarding the three clones are given in Figure 1.

Each construct carrying *Cf-9* with and without additional *Hcr9-9* genes was introduced separately into the susceptible tomato cultivar Moneymaker (Cf0), the tobacco cultivar Petite Havana, and the potato cultivar Maris Bard by Agrobacterium-mediated transformation.

Characterization of Tomato Transformants

To ascertain whether a disease resistance phenotype was introduced with the *Cf-9* genomic sequences, we inoculated primary tomato transformants carrying cosmid 2/9-34, cosmid

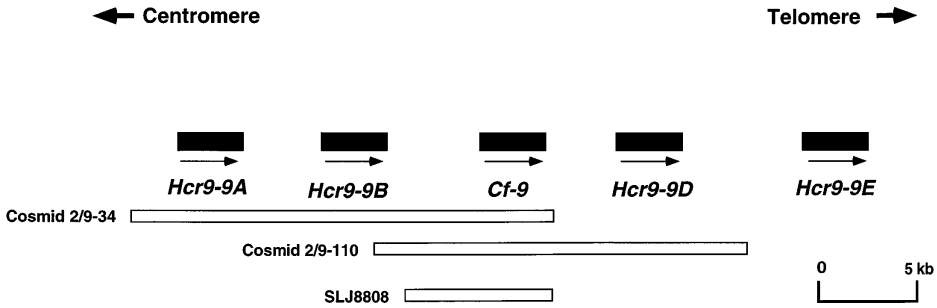

Figure 1. Genomic Region around *Cf-9* Indicating the Positions of the Four *Cf-9* Homologs (*Hcr9-9* Genes) and the Three Selected Clones.

The five solid boxes indicate the coding regions of *Cf-9* and the *Hcr9-9* homologs, and the horizontal arrow below each box indicates the direction of transcription (Parniske et al., 1997). The open boxes reveal the relative positions of cosmid 2/9-34, cosmid 2/9-110, and clone SLJ8808. The scale bar indicates distance in kilobases.

2/9-110, or SLJ8808 with race 5 of *C. fulvum.* Race 5 expresses the avirulence gene *Avr9* and is therefore unable to colonize plants possessing a functional *Cf-9* gene (De Wit and Spikman, 1982; De Wit et al., 1985). It is, however, able to colonize and sporulate on the cultivar Moneymaker, which was used for the transformation, because this cultivar lacks all known *Cf* resistance genes (Hammond-Kosack and Jones, 1994). The results of the pathogen inoculation tests with a total of 16 primary tomato transformants are summarized in Table 1. These data reveal that each clone enabled a susceptible tomato cultivar to resist a *C. fulvum* race that expressed the corresponding functional avirulence gene *Avr9.* T_2 progeny from a selected subset of the primary transformants that carried either one or two T-DNA insertions were retested for their resistance to *C. fulvum* race 5. The results of this second inoculation test using a total of six independent lines confirmed that the resistance trait is heritable. Only the data for construct SLJ8808 (*Cf-9* only) is shown in Table 1. Additional T_2 progeny from the same selected lines were also tested for their response to infection by *C. fulvum* race 5.9. This genetically engineered race does not produce functional Avr9 peptide and so is capable of sporulating on Cf9 tomato plants (Marmeisse et al., 1993). Tomato T_2 progeny possessing any of the *Cf-9* constructs were susceptible to race 5.9 (Table 1; data not shown).

To verify the *C. fulvum* inoculation tests, we injected each transformant with an IF preparation that contained the Avr9 peptide. In response to injection with this IF(Avr9$^+$), only tomato plants possessing a functional *Cf-9* gene develop a gray necrotic phenotype (Schottens-Toma and De Wit, 1988; Van Den Ackerveken et al., 1992; Hammond-Kosack and Jones, 1994). For these experiments, IF(Avr9$^+$) was prepared from either tomato leaves supporting a fully compatible Cf0–race 5 interaction or from transgenic tobacco plants engineered to secrete constitutively the Avr9 peptide into their apoplastic domains (Hammond-Kosack et al., 1994a). All of the tomato primary transformants and their progeny that gave a resistance phenotype to *C. fulvum* race 5 also exhibited a rapid gray necrotic response to IF(Avr9$^+$) challenge, whereas the *C. fulvum*–susceptible primary transformants and their progeny did not. When the IF(Avr9$^+$)-responsive plants were injected with the two different samples of IF(Avr9$^-$) described below, no macroscopic response appeared within 7 days. These new data, in conjunction with the earlier *Cf-9*–tagging experiment (Jones et al., 1994), prove that the specificity for recognition of the Avr9 peptide is conferred solely by the addition of a functional *Cf-9* gene product.

Characterization of Primary Tobacco and Potato Transformants Expressing Genomic *Cf-9* Sequences

Because *C. fulvum* is exclusively a pathogen of *Lycopersicon* species, only the Avr9 peptide challenge could be used to ascertain whether the *Cf-9* gene and its product are active in other plant species. To determine whether the correct specificity of action was retained in a heterologous system, plants were also injected with two types of IF lacking the Avr9 peptide. These were prepared from a compatible interaction involving tomato cultivar Moneymaker and *C. fulvum* race 5.9 (Marmeisse et al., 1993) and from untransformed Petite Havana tobacco plants. Most transformants exhibited a gray necrotic response to the two IF(Avr9$^+$) challenges and no macroscopic response to the two IF(Avr9$^-$) challenges. The results are summarized in Table 2 for the primary tobacco transformants and Table 3 for the primary potato transformants. All plants that were nonresponsive to the IF(Avr9$^+$) were found to lack the characteristic 7.6-kb BglII fragment that hybridizes with the *Cf-9* cDNA probe in DNA gel blot analyses (Jones et al., 1994) (data not shown). Figure 2 shows the typical appearances of tobacco and potato leaves 48 hr after injection with IF(Avr9$^+$) and IF(Avr9$^-$). When either IF was injected into the leaves of untransformed tobacco and potato plants, no macroscopic responses were evident (data not shown).

Table 1. Responses of Tomato Transformants Carrying the *Cf-9* Gene Alone or in Combination with Different *Hcr9-9* Genes to Two Races of *C. fulvum* and to IF Preparations Either Containing or Lacking the Avr9 Peptide

Primary Transformants	Number of Plants/Lines[a]	*C. fulvum* Race Inoculated[b]				IF Injection Tests[c]				Lowest Effective Avr9$^+$ IF Titer[d]
		Race 5 (Avr9$^+$)		Race 5.9 (Avr9$^-$)		Avr9 Containing: Cf-0/Race 5 IF or PH6201A IF		Avr9 Lacking: Cf-0/Race 5.9 IF or PH IF		
		R	S	R	S	+	−	+	−	
Controls										
Cf9	12/1	12	0	0	12	12	0	0	12	64
Cf0	12/1	0	12	0	12	0	12	0	12	—
Cosmid 2/9-34 (*Hcr9-9A, B,* and *Cf-9*)										
Primary transformants	1/4	3	1	ND[e]	ND	3	1	0	4	8–32
Cosmid 2/9-110 (*Cf9* and *Hcr9-9D*)										
Primary transformants	1/4	3	1	ND	ND	3	1	0	4	16–32
SLJ8808 (*Cf-9*)										
Primary transformants	1/8	5	3	ND	ND	5	3	0	8	8–32
T_2 Progeny										
Line F	48/1	18	6[f]	0	24	17	7[f]	0	24	32–64
Line H	48/1	16	8[f]	0	24	14	10[f]	0	24	16–32

[a]Number of plants per independent genotype (line) tested.

[b]R, fully resistant; S, fully susceptible, sporulation (day 14): tomato plants inoculated at the two-leaf growth stage.

[c]Tomato plants injected with a 1-in-2 IF dilution; (+), gray necrosis within 48 hr; (−), no response by 7 days. The IF dilution series tests were performed on eight T_2 plants at the three-leaf growth stage.

[d]Value given is the lowest dilution of IF to give visible necrosis. For example, 64 represents a 1-in-64 IF dilution. Dash indicates no response.

[e]ND, not determined.

[f]NPT assay negative, indicating that these T_2 plants had not inherited the T-DNA. The NPT$^-$ sibling plants were as susceptible to *C. fulvum* as the Cf0 controls. All R transformants were NPT$^+$.

The data presented in Tables 2 and 3 and Figures 2A and 2B clearly demonstrate that the tomato disease resistance gene *Cf-9*, expressed under the control of its endogenous tomato promoter and termination sequences, is functional in two other solanaceous species, namely, tobacco and potato, and that the specificity of the *Cf-9–Avr9* interaction is retained. In addition, the Avr9 responsiveness conferred by construct SLJ8808, containing the genomic *Cf-9* gene alone, demonstrates that the products of the neighboring *Hcr9-9* genes are not required for *Cf-9* gene function in either heterologous plant species.

Specific Features of the Avr9-Induced Gray Necrotic Response in Leaves of Potato and Tobacco Plants Expressing *Cf-9*

Homozygous lines were identified of tobacco transformants carrying a single T-DNA copy of the three different *Cf-9*–carrying T-DNAs. For all subsequent tobacco experiments, either one or two lines for each construct were selected. The detailed analysis of the response of potato leaves was conducted using a total of 23 of the original primary transformants that had either one or two T-DNA copies. These were vegetatively propagated by using shoot cuttings and tubers.

To determine the strength of the response to the Avr9 peptide challenge, we injected a dilution series of IF into fully expanded tobacco and potato leaves. Irrespective of the *Cf-9* construct, the tobacco leaves always responded to a much lower titer of IF than did either the tomato or potato leaves. Typically, a gray necrotic response to the Avr9 peptide derived from a compatible Cf-0–race 5 interaction was evident down to a dilution of 1 in 250 to 1 in 300, whereas in response to the IF(Avr9$^+$) from transgenic tobacco, gray necrosis was evident down to a dilution in the range 1 in 150 to 1 in 200. A summary of these data is given in Table 2. For potato leaves carrying the same three *Cf-9* constructs, the macroscopic responses were always within the range 1 in 4 to 1 in 64, as shown in Table 3. Transgenic tomato plants homozygous for *Cf-9* responded to a maximum dilution of 1 in 64 when the IF was prepared from a Cf-0–race 5 interaction and a 1 in 32 dilution when the IF came from transgenic tobacco (transformant 6201A) (Table 1; data not shown). The transgenic tomato plant responses to the IF(Avr9$^+$) were very similar to those obtained with the original homozygous Cf9 stock (Hammond-Kosack et al., 1994a; Hammond-

Kosack and Jones, 1994). These data indicate that the presence of the additional *Hcr9-9* genes in cosmids 2/9-34 and 2/9-110 in potato and tobacco neither potentiates nor diminishes the macroscopic response phenotype mediated by *Cf-9* to the Avr9 peptide.

The rate of gray necrosis development in *Cf-9*–expressing tobacco leaves in response to IF(Avr9$^+$) injection was much faster than in either tomato or potato leaves. Within 3 to 4 hr, confluent necrosis was evident in the injected tobacco leaf sections. This rapid response was evident down to an IF dilution titer of 1 in 64 (race 5 IF) and 1 in 32 (6201A IF) in all tobacco primary transformants (data not shown). Within the injected sections, the onset of host cell death, which was revealed by trypan blue stain accumulation (Keogh et al., 1980; Hammond-Kosack et al., 1996), commenced 2 to 2.5 hr after IF(Avr9$^+$) injection; by 3 hr after injection, all mesophyll cells were nonviable. The kinetics of cell death within the Avr9-injected tobacco leaf sections are shown in Figure 3. For the *Cf-9*–expressing potato leaves, the rate of host cell death and the subsequent rate of development of the gray necrotic response to Avr9 were comparable to the kinetics of these responses in tomato leaves (Figure 3). In leaves injected with the IF(Avr9$^-$), ~5 to 7% cell death was evident in each plant species (data not shown). Curiously, the epidermal cells within the Avr9-injected sections for each plant species never accumulated the trypan blue stain.

Supraoptimal opening of stomata has been demonstrated to be the main factor underlying the development of the Avr9-induced macroscropic gray necrosis phenotype in Cf9 tomato plants (Hammond-Kosack et al., 1996). Therefore, it was plausible that differences in the speed of the necrosis were due merely to interspecies differences in stomatal density. However, quantitative microscopic analysis revealed that there was a similar stomatal density in the injected leaves of the three plant species (data not shown). When leaf stomatal conductance was measured for the three plant species, the values obtained were again very similar (±10%; data not shown). Another plausible physical explanation for the fast response of tobacco leaves was that the intercellular airspaces were larger; therefore, a greater volume of IF could be injected. This would result in a higher Avr9 titer being delivered to a defined leaf area. However, a determination of fresh weight of leaf discs both before and after IF infiltration revealed only 10 to 15% greater airspace volume in tobacco compared with tomato and potato leaves (data not shown).

In tomato leaves, the macroscopic response to Avr9 challenge is always confined to the injected section; it has an initial uniform gray appearance that after 3 to 4 days turns brown (Hammond-Kosack and Jones, 1994). With the Avr9-challenged tobacco leaves, the gray necrosis was only confined to the injected area for 16 to 20 hr. Subsequently, confluent gray necrosis spread from the injected area into neighboring sections both proximal and distal to the site of injection. This spreading necrosis continued for up to 72 hr and often extended around the top of the leaf into the opposite leaf half, as shown in Figure 4A. The additional necrosis never entered the leaf petiole or adjacent stem tissue, and the Avr9 injection did not induce necrosis on other uninjected leaves of the plant.

Cf-9–expressing potato leaves injected with Avr9 peptide only developed a macroscopic reaction within the injected section. However, by 48 hr after injection, a black border formed around the injected area and thereafter delimited the injected tissue from the uninjected, as shown in Figure 2B. Microscopic analyses of the lesion margin revealed that

Table 2. Responses of Tobacco Transformants Carrying the *Cf-9* Gene Alone or in Combination with Different *Hcr9-9* Genes to Various IF Preparations Either Containing or Lacking the Avr Peptide

		IF Injection Tests[b]							
		Avr9 Containing: Cf-0/ Race 5 or PH6201A IF		Avr9 Lacking: Cf-0/ Race 5.9 or PH IF		Lowest Effective Avr9 IF Titer to Give Gray Necrosis on Each Transformant[c]			
Primary Transformants	Number of Plants/Lines[a]	+	−	+	−	1 in 50	1 in 75	1 in 100	1 in 125
Control									
Petite Havana (untransformed)	12/1	0	12	0	12	ND[d]	ND	ND	ND
Cosmid 2/9-34 (*Hcr9-9A*, *B*, and *Cf-9*)	1/10	7	3	0	10	1	2	2	2[e]
Cosmid 2/9-110 (*Cf-9* and *Hcr9-9D*)	1/6	5	1	0	6	3	1	0	1[e]
SLJ8808 (*Cf-9*)	1/9	7	2	0	7	2	2	1	2[e]

[a]Number of plants per independent genotype (line) tested.

[b]Tobacco plants injected with a 1-in-2 IF dilution when in flower; (+), gray necrosis within 48 hr; (−), no response by 7 days.

[c]Tobacco leaves injected with a serial dilution of the two different Avr9 IF preparations (source PH6201A) on leaves 10, 11, and 12 when each primary transformant had just come into flower.

[d]ND, not determined.

[e]The most active selected homozygous T_3 lines were cosmid 2/9-34, lines B (1 in 200) and I (1 in 150); cosmid 2/9-110, line B (1 in 150); and SLJ8808, line B (1 in 150). Leaves 10, 11, and 12 for each homozygous line were tested on six plants at the late-flowering stage. The lowest IF dilution at which visible gray necrosis was evident by 24 hr is given within brackets in the first sentence of this footnote.

cells in the mesophyll but not the epidermal layers had not only died but also had heavily accumulated a brown pigment (data not shown). A similar reaction was never evident at the margins of the Avr9-injected area in tomato and tobacco leaves.

The rapid reaction time and the spreading necrosis phenotype mediated by Cf-9/Avr9 in tobacco indicate a more responsive defense system than in tomato or potato. This difference, however, could be due to higher expression of the *Cf-9* transgene, greater Cf-9 protein abundance, or increased stability of the Avr9 peptide in the tobacco leaf apoplast. All of these possibilities still have to be rigorously explored.

Generation of Reactive Oxygen Species Precedes Cf-9/Avr9–Induced Cell Death in Both Tobacco and Potato

In tomato, the production of reactive oxygen species (ROS) precedes Cf-9/Avr9–dependent cell death by at least 9 hr (May et al., 1996). To ascertain the kinetics of ROS accumulation in Cf9 tobacco and potato leaves, nitro blue tetrazolium (NBT) staining was performed on discs cut at 15-min intervals after Avr9 injection from tobacco leaves and at 1-hr intervals for potato. In Cf9 tobacco, NBT staining was first evident by 75 min, whereas for Cf9 potato, it was detected by 3 hr. This NBT staining continued to be detected until the onset of cell death in both plant species (data not shown).

NBT staining could also be detected up to 15 mesophyll cells in advance of the spreading necrosis phenotype induced by Avr9 in Cf9 tobacco leaves between 16 and 60 hr after injection (data not shown). This latter finding suggested that excessive ROS accumulation, in particular, extracellular superoxide anion production, might be the cause of this runaway cell death in a manner analogous to that found for the Arabidopsis disease lesion mimic mutant *lsd1* (Jabs et al., 1996). Cell death in the *lsd1* mutant can be prevented by preinjecting leaves with an irreversible inhibitor of the mammalian plasma membrane NADPH oxidase complex called diphenylene iodonium (DPI). In an attempt to inhibit the spreading necrosis induced in Cf9 tobacco by Avr9, 5 and 10 μM DPI was injected repeatedly into the tissue surrounding the $Avr9^+$-injected section, namely, at 1, 6, and 15 hr after the original Avr9 injection. However, these treatments did not slow down the rate of necrotic spread compared with that observed in control leaves that had received three "buffer only" injections around the $Avr9^+$-injected section (data not shown). Similarly, injections with either superoxide dismutase or catalase in the tissue surrounding the Avr9-injected section failed to prevent spreading necrosis. We conclude from these pharmacological inhibitor experiments that other *Cf-9*/Avr9–induced physiological changes are probably the more likely cause of the spreading necrosis phenotype. Conceivably, nitric oxide (NO) contributes to this phenotype, because NO is now implicated in the plant defense response (Delledonne et al., 1998), and NO is also detectable by NBT staining.

Macroscopic Gray Necrotic Response to the Avr9 Peptide in Cf9 Tobacco Is Developmentally Regulated

Young tobacco seedlings with four leaves or less that carried any of the three *Cf-9* genomic clones did not respond macroscopically to Avr9 peptide challenge (1-in-2 dilution) under a range of environmental conditions examined. Plants at the five-leaf stage gave a weak response on leaf 5 at 48 hr after Avr9 injection, whereas by the six-leaf stage, confluent gray necrosis was obtained solely within the injected section. By the eight-leaf stage, when the apical meristem had just started to rise above soil level but the flower initials were

Table 3. Responses of Potato Transformants Carrying the *Cf-9* Gene Alone or in Combination with Different *Hcr9-9* Genes to Various IF Preparations Either Containing or Lacking the Avr9 Peptide

		IF Injection Tests[b]							
		Avr9 Containing: Cf-0/ Race 5 or PH6201A		Avr9 Lacking: Cf-0/ Race 5.9 or PH		Lowest Effective Avr9 IF Titer to Give Gray Necrosis on Each Transformant[c]			
Primary Transformants	Number of Plants/Lines[a]	+	−	+	−	1 in 4	1 in 8	1 in 16	1 in 32
Control									
Maris Bard (untransformed)	6/1	0	6	0	6	ND[d]	ND	ND	ND
Cosmid 2/9-34 (*Hcr9-9A*, *B*, and *Cf-9*)	1/3	3	0	0	3	0	1	1	1
Cosmid 2/9-110 (*Cf-9* and *Hcr9-9D*)	1/9	8	1	0	9	0	4	4	0
SLJ8808 (*Cf-9*)	1/11	10	1	0	11	2	1	6	1

[a]Number of plants per independent genotype (line) tested.

[b]Potato plants injected with a 1-in-2 IF dilution; (+), gray necrosis within 48 hr; (−), no response by 7 days.

[c]Fully expanded leaves on vegetatively derived progeny injected with a serial dilution of the Avr9 IF (source 6201A) when the plants were 60 cm high. Each plant contained either one or two T-DNA copies.

[d]ND, not determined.

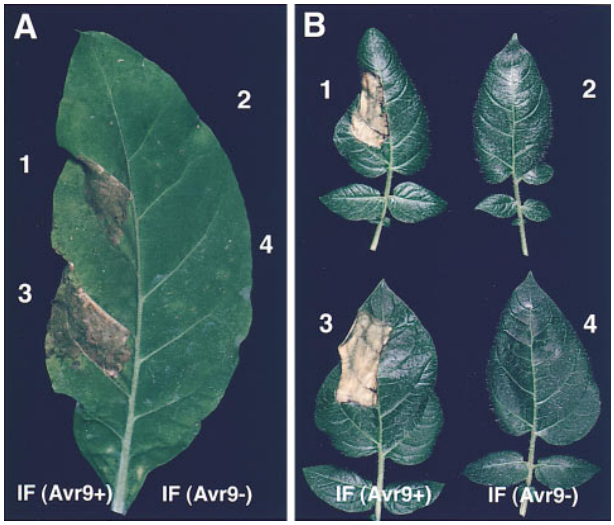

Figure 2. Avr9-Dependent Necrosis in Transgenic Cf9 Tobacco and Potato Leaves.

(A) Tobacco leaves after injection with four different types of IF. Two IFs contained the Avr9 peptide elicitor, IF(Avr9$^+$), and two lacked the Avr9 peptide, IF(Avr9$^-$). A gray necrotic response is evident only in the areas into which the Avr9 elicitor had been delivered, that is, sectors 1 and 3 but not 2 and 4. The different IF sources are as follows: 1, transgenic Petite Havana tobacco (SLJ6201A, *35S:Avr9*), producing the Avr9 peptide apoplastically (Hammond-Kosack et al., 1994a); 2, untransformed Petite Havana tobacco; 3, Cf0 (Moneymaker) tomato infected with *C. fulvum* race 5 (Avr9$^+$); and 4, Cf0 (Moneymaker) tomato infected with *C. fulvum* race 5.9 (Avr9$^-$). The photograph was taken 48 hr after IF injection.
(B) Potato leaves injected with the same four IF preparations, as described above, and photographed after 48 hr.

not yet visible, the full response to Avr9 (as described above) was evident within 24 hr. In potato plants as in tomato plants, there was no observable developmentally regulated pattern to the response to Avr9 in leaves.

Severity of the Gray Necrotic Response to Avr9 in Tobacco Leaves Is Enhanced by Sequential Challenges

When *Cf-9*–expressing tobacco plants were sequentially challenged with Avr9 peptide at weekly intervals, the macroscopic response to each subsequent challenge was heightened. For example, if plants at the five-leaf stage had one section injected with Avr9 at a 1-in-2 dilution or with healthy control IF, and 7 days later the next leaf above was injected, the response of the plant that had already responded to the earlier Avr9 challenge was significantly increased. Within 48 hr of the second Avr9 challenge, the entire leaf half collapsed, whereas without prior Avr9 infiltration, gray necrosis only developed within the injected area, as shown in Figures 4B and 4C. This response resembles that normally observed in plants where systemic acquired resistance (SAR) has been established by an initial biotic necrosis-inducing challenge. SAR leads to heightened disease resistance in subsequently challenged leaves, manifested as a faster development of accompanying necrosis and a more rapid containment of the pathogen (Ryals et al., 1996).

Avr9 Transgene Causes Either Developmentally Conditioned Seedling Lethality or a No-Seed-Germination Phenotype When Crossed to Cf9 Tobacco

When Cf9 tomato plants are crossed to tomato plants constitutively secreting the Avr9 peptide into their apoplastic domains, a seedling lethal phenotype occurs within 14 days of seed sowing (Hammond-Kosack et al., 1994a). To determine whether a similar phenotype occurs in tobacco, primary transformants heterozygous for a single *Cf-9* T-DNA copy were crossed as the pollen donor to tobacco plants homozygous for the *35S:Avr9* construct (SLJ6201, transformant A) (Hammond-Kosack et al., 1994a). The (*35S:Avr9* × Cf9) F_1 tobacco seedlings developed at an identical rate for the first 7 days after seed sowing (i.e., for the first 2 days after root radicle emergence), even though the seedlings segregated 1:1 for the *Cf-9* T-DNA. The first visible effects of

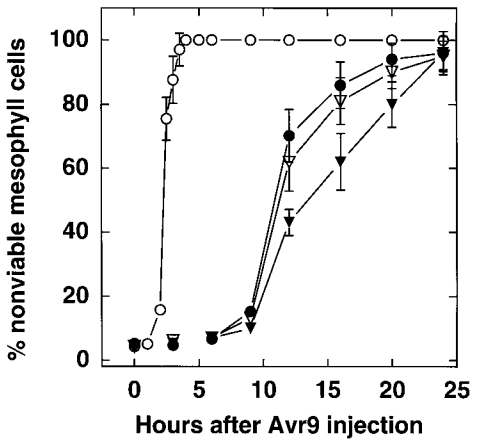

Figure 3. Kinetics of Host Cell Death Induced in Tomato, Tobacco, and Potato Leaves Expressing *Cf-9* after Injection with the Avr9 Elicitor.

Plants grown under high-light and high-temperature greenhouse conditions are indicated by open circles (tobacco) and closed circles (tomato). Plants grown at a lower temperature and in a lower light growth cabinet are indicated by open triangles (potato) and closed triangles (tomato). The percentage of death of mesophyll cells was assessed by trypan blue staining and microscopic observation. In leaves injected with IF(Avr9$^-$), ~5% cell death was evident in each species throughout the time course. Bars indicate standard deviation.

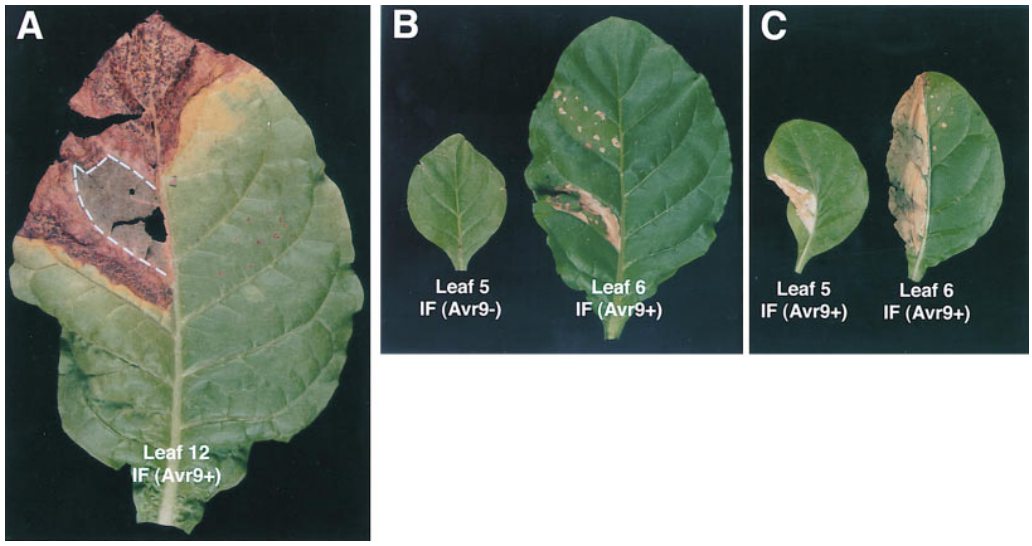

Figure 4. Avr9 Peptide Injection into Cf9 Tobacco Leaves Causes a Developmentally Regulated Spreading Necrotic Reaction That Can Be Enhanced by Sequential Avr9 Challenges.

(A) Leaf 12 taken from a flowering Cf9 tobacco plant (SLJ8808J) 72 hr after a single leaf section had been injected with a 1-in-100 dilution of transgenic tobacco Avr9 IF. The injected area is delimited by the dashed white lines.

(B) Leaves 5 and 6 taken from two different young tobacco plants that had each received a different IF injection treatment. Leaf 5 was injected with control IF(Avr9$^-$). Seven days later, two areas on leaf 6 were injected with a 1-in-2 dilution of Avr9$^+$ tobacco IF.

(C) Leaf 5 infected with a 1-in-2 dilution of Avr9$^+$ tobacco IF. Seven days later, two areas on leaf 6 were injected with the identical Avr9$^+$ IF preparation.

Photographs in **(B)** and **(C)** were taken 7 days after the second injection. The smaller size of leaf 6 in **(C)** compared with leaf 6 in **(B)** was consistently observed. This indicates that the expansion rate of young tobacco leaf tissue is diminished as a consequence of Cf-9/Avr9–mediated defense responses.

the combined *Avr9* and *Cf-9* transgenes were evident between 7 and 8 days after seed planting. Both root growth and cotyledon expansion ceased from day 7 onward, and by day 9, the cotyledons began to turn chlorotic, as revealed in Figure 5A and Table 4, crosses 1 and 2. F_1 seedlings that remained green and healthy had not inherited the *Cf-9* sequence, as determined by polymerase chain reaction (PCR) analysis using *Cf-9*–specific primers on pooled samples (total of $n = 60$ from six separate crosses; data not shown). As anticipated, when T_2 tobacco plants homozygous for the *Cf-9* gene were crossed as the male parent to plants homozygous for the *Avr9* transgene, there was 100% seedling lethality (data not shown). An identical F_1 seedling lethal phenotype occurred when transgenic tobacco lines carrying either cosmid 2/9-34 or cosmid 2/9-110 (i.e., the *Cf-9* gene in combination with various *Hcr9-9* genes) were crossed to the same Avr9-expressing plant (data not shown).

In the reciprocal crosses using the Cf9 lines as the female parent and the homozygous Avr9 line as the pollen donor, both pod set and size were normal (data not shown), but when the F_1 seed was plated, very few seeds germinated (Figure 5B and Table 4, crosses 3 and 4). To test whether the no-seed-germination phenotype was a seed autonomous trait, tobacco plants heterozygous for the *Cf-9* transgene (SLJ8808) were crossed as the female parent to plants heterozygous for the *Avr9* transgene. The four predicted genotypic outcomes arising from this cross are depicted in Figure 6; only 25% of the F_1 progeny inherit both transgenes (genotype A). However, as the level of seed germination is well below 50% (Table 4, crosses 5 and 6), it must be concluded that this trait is conferred nonautonomously. When the F_1 seed was plated on kanamycin, slightly >50% of the germinating seed were found to be kanamycin resistant. Additional PCR analysis showed that the kanamycin-resistant progeny carry either the *Cf-9* T-DNA (genotype B) or the *Avr9* T-DNA (genotype C) in approximately equal numbers (data not shown). These data indicate that the genotype of the zygote is not the primary cause underlying the no-seed-germination phenotype.

Coexpression of the *nahG* Transgene in Cf9 Tobacco Both Modifies the Macroscopic Response to Avr9 Peptide Injection and Restores Germination to Cf9 × *35S:Avr9* F_1 Seeds

SA has been demonstrated to be required for resistance mediated by *R* genes of the NBS/LRR class, such as the tobacco *N* gene against tobacco mosaic virus (TMV), Arabi-

dopsis *RPP* genes against *Peronospora parasitica*, and the Arabidopsis *RPS2* gene that confers resistance to *Pseudomonas syringae* pv *maculicola* (Gaffney et al., 1993; Delaney et al., 1994). SA is also known to potentiate several features associated with disease resistance, including the oxidative burst, pathogenesis-related gene induction, and host cell death (reviewed in Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). To investigate whether a reduction in the levels of inducible SA could modify various *Cf-9*– and Avr9-dependent responses, we introduced a *35S:nahG* transgene (transformant line SLJ7321C) (Brading, 1997) by sexual crossing into several of the *Cf-9* tobacco lines. This 7321C line compromises tobacco *N* gene–mediated resistance to TMV (Brading, 1997) in a manner analogous to that originally reported by Gaffney et al. (1993).

Introduction of the *35S:nahG* gene into each Cf9 tobacco line resulted in both a reduction in the Avr9 dilution end point, at which a macroscopic gray necrotic response was evident, and a lessening of the subsequently induced spreading brown necrotic reaction (Table 5). In Cf9 tomato plants, coexpression of the identical *nahG* transgene caused a reduction in Avr9 responsiveness within the injected section (Brading, 1997). To establish whether SA potentiated the macroscopic gray necrosis response, we added 500 μM SA to different titers of IF(Avr9$^+$) derived from transgenic tobacco just before injection of tobacco lines carrying construct SLJ8808. At subthreshold titers of transgenic tobacco-derived Avr9 IF, namely, 1 in 200, 250, and 300, the addition of SA caused the development of macroscopic gray necrosis within the injected section with or without additional spreading necrosis (Table 6). When added to IF(Avr9$^+$) titers capable of causing gray necrosis at the injection site but unable to initiate spreading necrosis from it (i.e., 1 in 150), SA caused the initiation of extensive, spreading gray necrosis (Table 6). However, the addition of 500 μM SA to a very high IF(Avr9$^+$) titer (i.e., 1 in 2) caused neither an obvious enhancement of the speed of the response nor the extension of the spreading necrosis into the petiole and adjacent stem tissue or uninjected leaves (data not shown).

When a *Cf-9* and *35S:nahG* double heterozygote plant was used as the female parent in a cross to a line homozygous for the *35S:Avr9* transgene, 100% germination of the resultant F_1 progeny occurred (Figure 5C) compared with the no-germination phenotype evident when a heterozygous *Cf-9* individual that lacked *35S:nahG* was used as the female parent (Figure 5B). However, by 5 days after root radicle emergence, the onset of seedling lethality occurred in 50% of the progeny, and this then proceeded in an identical fashion to that produced in F_1 seedlings derived from the reciprocal *Cf-9* (male) × *35S:Avr9* (female) cross (Figure 5D and Table 4, crosses 7 and 8). When the *35S:Avr9* parent was heterozygous, 25% of the progeny exhibited seedling lethality (data not shown). The F_1 progeny obtained from an *Avr9* × *nahG* double heterozygote female × *Cf-9* homozygote male cross all germinated, but subsequently the wild-type seedling lethal phenotype occurred in ~50% of the progeny (Table 4, crosses 9 and 10). In contrast, when a *Cf-9* homozygous plant was used as the female parent and an *Avr9* × *nahG* F_1 plant was used as the male line, the no-seed-germination phenotype was again evident (Table 4, crosses 11 and 12). The ability of the *35S:nahG* transgene to eliminate completely the no-seed-germination phenotype only when introduced via the female parent indicates that elevated SA levels in maternal tissue are either directly or indirectly causally involved.

DISCUSSION

The isolation of numerous plant disease resistance genes, of which each is effective against a single pathogenic microorganism, has revealed the existence of at least four distinct classes of race-specific *R* gene sequences (Baker et al., 1997; Hammond-Kosack and Jones, 1997). One question of immense practical significance to disease control is whether members from each *R* gene class will function when transferred between sexually incompatible plant species. Before the cloning of *R* genes, plant breeders were restricted in the plant genotypes that could be utilized as a source of novel resistance traits. In this study, we demonstrate that the tomato *Cf-9* gene, which codes for an extracytoplasmic LRR protein, confers responsiveness to the corresponding fungal avirulence gene product, Avr9, in both tobacco and potato. These findings now extend to three the repertoire of *R* gene classes in which individual members have been found to function in heterologous plants. Previous studies have shown that tomato *Pto* is a unique member of the serine/threonine protein kinase class that confers resistance to *avrPto*-expressing *P. syringae* in *N. tabacum* and *N. benthamiana* (Rommens et al., 1995; Thilmony et al., 1995). Whitham et al. (1996) demonstrated that the tobacco *N* gene, which is a member of the TIR/NBS/LRR class, confers TMV resistance in tomato.

Avr9 responsiveness conferred on tobacco and potato by the *Cf-9* gene indicates the presence in these species of functional homologs of all of the molecular partners required for *Cf-9* gene product function in tomato. Because *Cf-9*–mediated resistance in tomato requires the presence of at least two additional tomato genes, which have been identified by genetic mutation analysis and called *Rcr-1* and *Rcr-2* (Hammond-Kosack et al., 1994b), these must also be functional in both potato and tobacco. Comparable results obtained with the minimal genomic *Cf-9* construct (SLJ8808) and the two cosmid clones that contained *Cf-9* plus the neighboring highly related *Hcr9-9* gene sequences (Parniske et al., 1997) also indicate that the gene products of *Hcr9-9* A, B, and D are probably not required for *Cf-9* function. When *Cf* gene sequences were used to probe DNA isolated from various solanaceous species, numerous *Cf*/*Hcr* gene homologs were detected (Dixon et al., 1996; Kooman-Gersmann

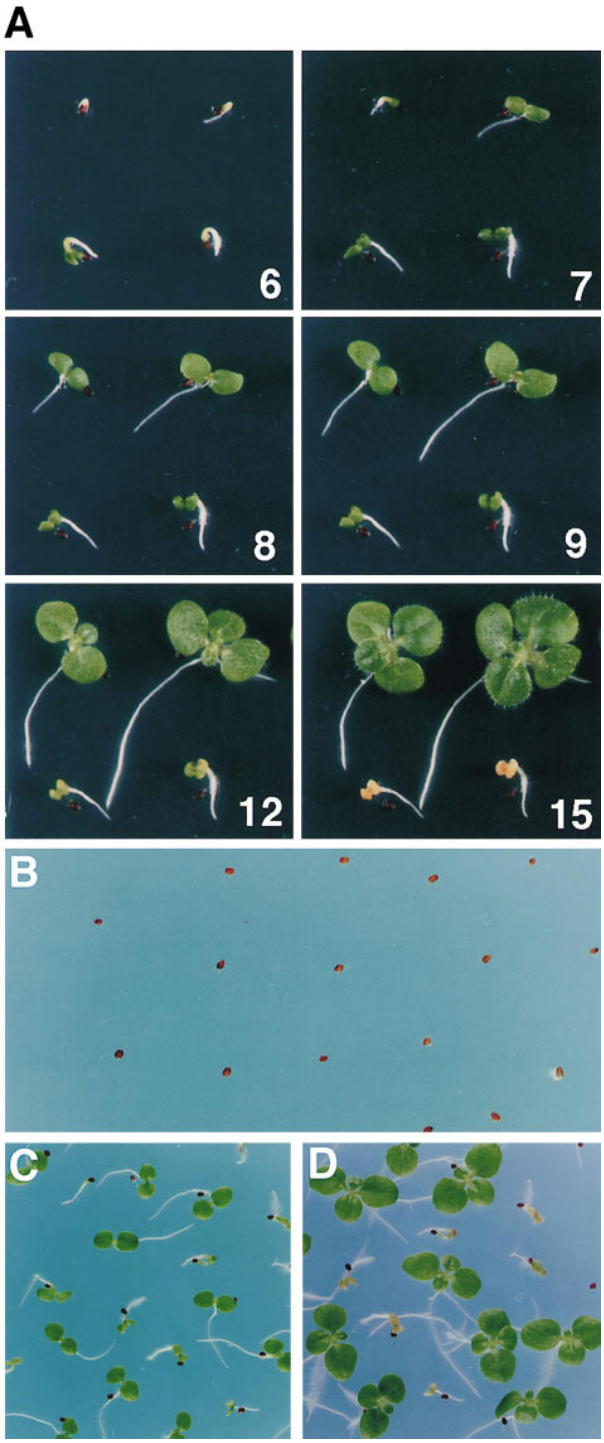

Figure 5. Development of the Necrotic Lethal Phenotype in Tobacco Seedlings from the Cross Petite Havana SLJ6201A (*35S:Avr9*; Hammond-Kosack et al., 1994a) and Petite Havana *Cf-9* (Cosmid 2/9-34B) Heterozygote.

(A) A time course of F_1 progeny growth for the period of 6 to 15 days after seed planting. Progeny were derived from a cross in which the female parent expressed Avr9 and the Cf9 plant was the pollen donor. In the six sections, the same four seedlings have been photographed. The number in the lower right corner of each photograph indicates the day after seed planting. From day 8 onward, retarded growth and eventual death of the two lower seedlings that had inherited both the *Avr9* and *Cf-9* T-DNAs are evident.

(B) Result of the Cf9 (female) × *35S:Avr9* (male) cross photographed at 9 days after seed planting revealing the no-seed-germination phenotype.

(C) and **(D)** The introduction of a *nahG* transgene encoding for salicylate hydroxylase (Gaffney et al., 1993) into the female Cf9 parent (i.e., SLJ7321C [*35S:nahG*] × Cf9 F_1) before pollination by the Avr9 homozygous line resulted in the full restoration of seed germination. However, the presence of the *nahG* transgene did not alter the subsequent rate of development of the seedling lethal phenotype. Photographs were taken at 9 and 15 days after seed planting in **(C)** and **(D)**, respectively.

et al., 1996). It will be interesting to evaluate the function of *Cf-9* in other than solanaceous plant species, for example, Arabidopsis and rice.

Avr9 Responsiveness in Cf9 Tobacco Is Distinct

A consistent feature evident throughout the analysis was the dissimilarity in the speed, severity, and type of response induced by Avr9 in the Cf9 tobacco compared with tomato and potato. The only exceptions were the constant gray appearance of the initial response in all three species and the lack of cell death in the L1 (epidermal) cell layers. One particularly striking difference in tobacco was the speed of the induced hypersensitive response (HR), with >80% of cell death occurring within 3 hr of Avr9 injection. This HR phenotype is also considerably faster than has been reported to occur in tobacco leaves after leaf infiltration with other microbial elicitors, for example, fungal elicitins, bacterial harpins, or avirulent bacteria at high titer (>10^8 colony-forming units), that typically develop within 12 to 16 hr. The reason for this highly reproducible rapid macroscopic response is unclear, but it may indicate that more efficient *Cf-9* controlled signaling or effector partners must be present in *N. tabacum* than the other two species.

The spreading nature of the macroscopic HR to Avr9 in Cf9 tobacco also contrasts with the restricted response evident after bacterial or elicitor challenge of tobacco. Its occurrence and the detection of NBT staining ahead of the advancing necrotic edge suggest that plant cell protection mechanisms, in particular, against the Cf-9/Avr9–induced oxidative burst, have been overloaded or have not evolved to cope with such a stress. Elevated ROS production underlies the development of the spreading necrosis evident in the Arabidopsis disease lesion mimic mutant *lsd1* (Jabs et

Table 4. Reciprocal Crosses between *Cf-9*– and *Avr9*-Expressing Tobacco Give Two Different Outcomes: A Seedling Lethal or a No-Germination Phenotype[a]

			Progeny Phenotype				
Cross	Female Parent[b]	Male Parent	No. of Seeds Not Germinating	No. of Necrotic Seedlings	No. of Healthy Seedlings	Total No. of Seed Sown	Percentage of Germination
1	Avr9 (homo)	Cf9 (8808B) (het)	0	167	174	341	100
2	Avr9 (homo)	Cf9 (8808J) (het)	0	186	203	389	100
3	Cf9 (8808B) (het)	Avr9 (homo)	382	5	0	392	2.5
4	Cf9 (8808J) (het)	Avr9 (homo)	378	16	0	394	4.0
5	Cf9 (8808B) (het)	Avr9 (het)	331	4	228	563	41.2
6	Cf9 (8808J) (het)	Avr9 (het)	307	11	151	469	34.3
7	Cf9 (8808B) × *nahG* (SLJ7321C)	Avr9 (homo)	6	179	193	378	98.4
8	Cf9 (8808J) × *nahG* (SLJ7321C)	Avr9 (homo)	9	161	173	343	97.4
9	Avr9 (homo) × *nahG* (homo)	Cf9 (8808B) (homo)	0	141	158	299	100
10	Avr9 (homo) × *nahG* (homo)	Cf9 (8808J) (homo)	0	173	185	358	100
11	Cf9 (8808B) (homo)	Avr9 (homo) × *nahG* (homo)	413	3	12	428	3.6
12	Cf9 (8808J) (homo)	Avr9 (homo) × *nahG* (homo)	379	5	7	391	3.1

[a]The seedling lethal phenotype can be reversed when a *nahG* transgene is present in the Cf9 female parent.
[b]homo, homozygous for T-DNA; het, heterozygous for T-DNA.

al., 1996). However, the fact that this spreading necrosis could not be reversed by DPI, superoxide dismutase, or catalase means that the underlying cause of the spreading necrosis remains unclear. In Cf9 potato, like tomato, spreading necrosis was never induced. Recent work implicating NO in plant defense (Delledonne et al., 1998) and the fact that NO can be revealed by NBT staining suggest that NO could be contributing to this phenotype. Because microscopic analysis of the lesion margins in potato revealed severe tissue darkening, the possibility exists that additional induced responses in potato effectively halted progressive necrosis.

The inability of young Cf9 tobacco plants to respond macroscopically to Avr9 injection was unexpected. In tomato, a macroscopic gray necrotic response to Avr9 is always evident from the cotyledon stage of growth onward. Also at the cellular level, Cf-9/Avr9–specific events are recognized to occur even in these young tobacco plants. For example, an influx of Ca^{2+} ions into Cf9 tobacco leaf guard cells is specifically induced within 5 min after the addition of Avr9 (M. Blatt, K.E. Hammond-Kosack, and J.D.G. Jones, unpublished data). Therefore, in the leaves of young tobacco plants, either cellular protection mechanisms are adequate to cope with the induced responses that in older leaves cause necrosis or only certain components of the Cf-9/Avr9–mediated defense response that are insufficient to lead to cell death occur in the young plants.

We noted a pronounced heightening of Avr9 responsiveness by sequential challenges. Also, coinjection of SA and Avr9 enhances the response (Table 6). When SAR has been established after an initial biotic necrosis-inducing challenge, the tobacco *N* gene functions more rapidly to close down pathogen spread, and the HR lesions associated with virus containment are smaller. SAR plants exhibit enhanced disease resistance, manifested as both a reduction in the level of pathogen infection and the level of accompanying necrosis (Ryals et al., 1996). The systemic enhancement of Avr9 responsiveness after more than one challenge with elicitor is consistent with a role of SA, although alternative or additional molecules may also play a role.

The Cf9 tobacco data reveal that Avr9 responsiveness is occasionally nonexistent, sometimes weak, but frequently exceeds the macroscopic reaction evident in tomato. In addition, the strength of the response can also be heightened by elevating a single biochemical component, namely, SA. These results reinforce the idea that the network of signaling events controlling R–Avr—mediated defense induction contains both positive and negative checkpoints to achieve fine-tuning. Potentially, therefore, when *R* genes are expressed in more distantly related plant species, some of these endogenous checkpoints may not function correctly, and this results in similarly dramatic fluctuations in function at distinct stages of plant development and/or R protein nonfunctionality in specific plant organs.

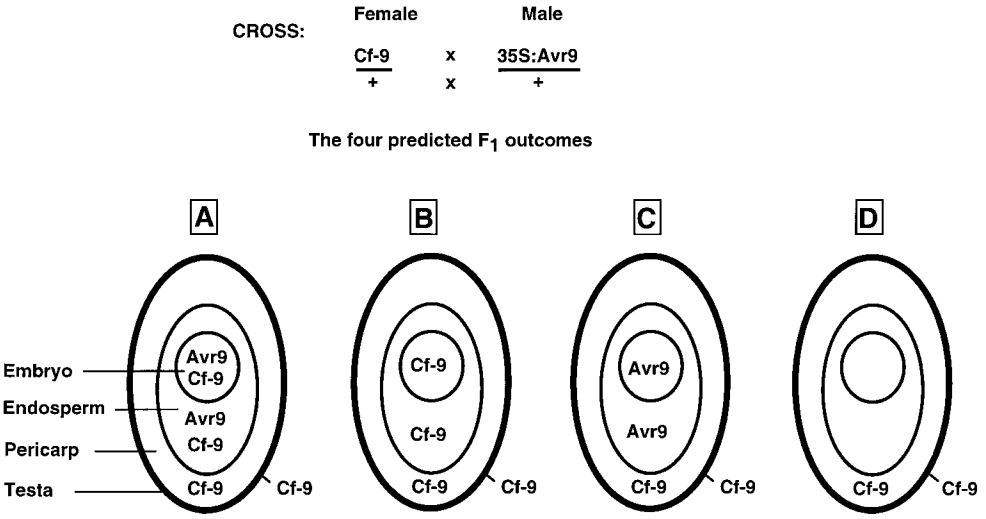

Figure 6. Four Predicted Genotypes from the Cross *Cf-9* Heterozygous Female Parent × *35S:Avr9* Heterozygous Male Parent.

Indicated are the genotypes of the sexually derived embryo and endosperm tissues and the maternally derived pericarp and testa tissues. It is hypothesized that if the no-seed-germination phenotype is seed autonomous, then only 50% of the tobacco seed will germinate, because all seeds express Cf-9 in the maternally inherited tissues, but only genotypes B and D also lack *Avr9* expression somewhere in the seed. If, however, the phenotype is a seed nonautonomous trait, then a reduction in the germination of genotype classes B and D will also occur in the F_1 progeny.

Table 5. The Presence of a *nahG* Transgene Partially Compromises Avr9-Induced HR in Cf9 Tobacco Leaves

	Lowest Effective Avr9 IF Titer to Give Gray Necrosis[a]	
	IF Source	
Genotype	Cf-0/Race 5	PH6201A
Cosmid 2/9-34 Line B × PH	150[b]	100
Cosmid 2/9-34 Line B × *nahG*	75	25
SLJ8808 Line B × PH	100	50
SLJ8808 Line B × *nahG*	50	15
SLJ8808 Line J × PH	125	75
SLJ8808 Line B × *nahG*	75	25

[a] Injected leaves 10, 11, and 12 on tobacco plants when in flower. Each experiment was repeated four times using a minimum of four plants per treatment per genotype.
[b] Serial dilution of IF where 50 = 1 in 50.

Cf-9/*Avr9*–Mediated No-Seed-Germination Phenotype in Tobacco

Reciprocal crosses established that the no-seed-germination phenotype observed in F_1 progeny derived from a cross between a *Cf-9*–expressing female parent and a *35S:Avr9*–expressing male pollen donor is a seed nonautonomous trait. Interestingly, the introduction of the *35S:nahG* gene into the maternal line leads to the restoration of seed germination. This clearly indicates that increased SA levels are causally involved in the establishment of the phenotype, either directly or indirectly. In Figure 7, three plausible explanations for the no germination phenotype are illustrated. They are as follows.

(1) The small size of the Avr9 peptide (28 amino acids) and its targeted secretion to outside of the plant cell mean that Avr9 peptide could easily diffuse through plant cell walls and so result in elevated levels of Avr9 throughout the developing tobacco pod. Thus, *Cf-9*$^+$ *Avr9*$^-$ seeds would also probably come into contact with considerable concentrations of Avr9 and activate defense responses. Similarly, in the *Cf-9*$^-$ *Avr9*$^-$ and *Cf-9*$^-$ *Avr9*$^+$ seeds, the presence of Cf-9 in the maternally derived endosperm and seed coat tissue would also permit interaction with any diffusible Avr9, generated either within the seed or from neighboring seed, again activating a defense response.

(2) From seeds with the genetic constitution *Cf-9*$^+$ *Avr9*$^+$, toxic reaction products from the incompatible interaction could diffuse to neighboring seeds and influence their devel-

opment. SA, ROS, lipid peroxides, and ethylene are all potential candidates known to be synthesized in incompatible reaction mediated by *Cf-9*/*Avr9* (Hammond-Kosack et al., 1996; May et al., 1996). However, because both pod size and 1000-seed weight appeared the same in all crosses, premature embryo death appears to be the least likely explanation for the no-seed-germination phenotype.

(3) The *Cf-9*/*Avr9*–induced oxidative burst, which is likely to be of considerable magnitude in the developing tobacco pod, induces the oxidative cross-linking of cell wall proteins that overstrengthen the seed testa and other cell walls throughout the developing seed, and this inhibits the necessary cell expansion for seed germination to occur. SA is known to potentiate the oxidative burst (Shirasu et al., 1997), and so by lowering SA levels in the maternally inherited tissue of the nahG plants, the oxidative burst could be sufficiently reduced to permit successful seed germination. Tomato fruits, in contrast to tobacco pods, contain seeds individually surrounded by gelatinous material, and so each seed is well isolated from its neighbors and concentrations of Avr9 and defense and signaling molecules would be lower. The fact that the original transposon-tagging experiment to isolate the *Cf-9* gene was done using the Cf9 line as the female parent with the *35S:Avr9* gene provided by the pollen donor (Jones et al., 1994) and the data of Hammond-Kosack et al. (1994a) on reciprocal crosses of *35S:Avr9* and Cf9 tomato show that the no-seed-germination phenomenon does not occur in this species.

Biotechnological Exploitation of *Cf-9*/*Avr9* for Broad-Spectrum Disease Control

Although *C. fulvum* is exclusively a pathogen of tomato, the availability of the cloned *Cf-9* and *Avr9* genes to induce a rapid HR should allow novel strategies of resistance to be engineered in heterologous plant species. Of particular importance is the fact that all of the Cf9 tobacco and potato transformants appeared to develop normally in the absence of Avr9 challenge and that the specificity of the response to Avr9 was retained. Although the responses induced by the wild-type *Cf-9* and *35S:Avr9* genes lead at best to severe macroscopic necrosis and at worst in the appropriate sexual crosses to developmentally regulated seedling death, various methods have already been devised or suggested to utilize this gene combination to control destructive pathogens. For example, several weak alleles of each gene are known that possibly could be used to engineer plants that consitutively express a very weak defense response throughout the plant (Hammond-Kosack et al., 1994b; Kooman-Gersmann et al., 1997). A pathogen-inducible promoter system has been suggested to limit defense gene induction to the precise site of pathogen (De Wit, 1992). Alternatively, by combining three transgenes into one plant line, with the *Cf-9* gene inactivated by a maize *Dissociation* transposable element, a stabilized maize *Activator* transposase source, and the *35S:Avr9* transgene, enhanced resistance to various fungal pathogens can be obtained (Jones et al., 1995). In this system, termed genetically engineered acquired resistance (GEAR), somatic excision of the *Dissociation* transposon restores *Cf-9* function, which results in the localized activation of plant defense responses because the apoplastically produced Avr9 peptide is now recognized.

In conclusion, the ability to express different classes of cloned *R* genes in any plant species provides the possibility of pyramiding various engineered *R–Avr* combinations, which may operate via different mechanisms. However, this approach may generate inherent new risks for the future. One potential problem is the likelihood of a significant shift in pathogen populations that could render ineffective these novel resistances simultaneously. To minimize this possibility, a temporal or spatial delimitation of the period over which these engineered *R*/*Avr* genes operate may be required. This type of strategy, borrowed from nature, has already provided tomato breeders, unknowingly, with durable disease control for *C. fulvum* by using *Cf-9* in combination with the linked *Hcr9-9* genes (Parniske et al., 1997).

METHODS

Genomic *Cf-9* Clone Isolation

A binary cosmid genomic DNA library prepared from plants homozygous for *Cf-2* and *Cf-9* (Dixon et al., 1996) was screened for the presence

Table 6. Coinjection of Avr9 IF with SA into Wild-Type Cf9 Lines Enhances the Macroscopic Necrosis Response

	6201A IF Alone[a]							6201A IF plus 500 μM SA[a,b]						
Genotype	50[c]	75	100	150	200	250	300	50	75	100	150	200	250	300
SLJ8808B	Gs[d]	Gs	Gs	G	—	—	—	Gs	Gs	Gs	G	—	—	—
SLJ8808J	Gs	Gs	Gs	G	—	—	—	Gs	Gs	Gs	G	—	—	—

[a] Each experiment was repeated four times using a minimum of four plants per treatment per genotype.

[b] Injected leaves 10, 11, and 12 on tobacco plants when in flower. Leaves were injected with a serial dilution of IF where 50 = 1 in 50.

[c] SA added to IF just before injection; 500 μM SA alone gave no macroscopic response.

[d] Gs, confluent gray necrosis and spreading necrosis by 16 hr; G, confluent gray necrosis by 16 hr; (—), no symptoms.

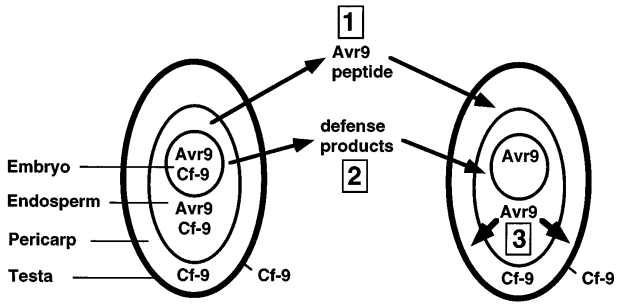

Figure 7. Model Illustrating the Three Most Likely Explanations for the Seed Nonautonomous *Cf-9–35S:Avr9*—Mediated No-Seed-Germination Phenotype.

Explanation 1 is that the Avr9 peptide diffuses between developing seeds, explanation 2 is that defense reaction products diffuse between developing seeds, whereas explanation 3 is that Avr9 peptide diffuses from embryo and endosperm tissue into the pericarp and testa tissue. For full details, see text.

of *Cf-9* by using polymerase chain reaction (PCR) in pools followed by hybridization with the 5′ end of the *Cf-9* cDNA clone; clones carrying *Cf-9* sequences were further characterized by restriction enzyme digests and DNA gel blot analyses (Jones et al., 1994; Thomas et al., 1995). Subsequently, a 7.5-kb PstI subfragment was identified that carried the intact *Cf-9* gene. This was cloned into the kanamycin resistance–conferring binary vector SLJ7291 (http://www.uea.ac.uk/nrp/jic/sl.htm) to produce the clone SLJ8808. All DNA techniques used in this study were performed according to Sambrook et al. (1989).

Plant Transformation and Selection of Single-Copy T-DNA Insertion Lines

Transformations were conducted using the kanamycin-sensitive tomato cultivar Moneymaker (*Lycopersicon esculentum*), tobacco cultivar Petite Havana (*Nicotiana tabacum*), and potato cultivar Maris Bard (*Solanum tuberosum*). The binary vector cosmids 2/9-34 and 2/9-110 (Thomas et al., 1995) and binary clone SLJ8808 were mobilized into *Agrobacterium tumefaciens* LBA4404. Transgenic plants were regenerated by the method of Fillatti et al. (1987) for tomato, Horsch et al. (1985) for tobacco, and Kumar et al. (1996) for potato. Up to 11 independent transformants of each species–construct combination were selected for further analysis. To select the subset of primary tobacco transformants carrying single T-DNA integration sites, T_2 progeny seed was plated on Murashige and Skoog (1962) medium containing 300 μg/mL kanamycin sulfate, and the ratio of kanamycin-resistant to sensitive seedlings was determined. Subsequent DNA gel blot analysis of these selected lines was used to determine which lines carried a single-copy T-DNA insertion. All potato primary transformants were analyzed by DNA gel blot analysis using a probe specific to the neomycin phosphotransferase gene.

Plant Growth Conditions

Tomato and tobacco plants were grown in Levington's M3 compost (Levington Horticulture Ltd., Fisons, Ipswich, UK) in a greenhouse at temperatures between 22 and 27°C in the light and 12 to 16°C in the dark. Sixteen hours of light were supplied at a photon flux density of 300 to 650 μE m^{-2} sec^{-1} at the plant surface, and the relative humidity was between 70 and 80%. Potato plants were grown in Levington's M3 compost plus 15% [v/v] fine gritstones in a growth chamber maintained at 16°C with a 16-hr day length, where light was supplied at a photon flux density of 350 μE m^{-2} sec^{-1}.

Cladosporium fulvum Pathogenicity Tests

Tomato transformants were assayed for disease resistance by using *C. fulvum* races 5 and 5.9 (Marmeisse et al., 1993), exactly as described by Hammond-Kosack and Jones (1994). When inoculated, the plants were at either the cotyledon or three-leaf growth stage.

Isolation and Injection of Avr Race-Specific Elicitors

Four types of elicitor preparations were recovered from leaf airspaces by the procedure of De Wit and Spikman (1982). Intercellular washing fluids (IFs) containing or lacking Avr9, IF(Avr9$^+$), or IF(Avr9$^-$), respectively, were recovered from the leaves of untransformed Moneymaker tomato heavily infected with either *C. fulvum* race 5 or race 5.9 (Marmeisse et al., 1993), respectively. From transgenic tobacco plants engineered to constitutively produce the Avr9 peptide in the apoplast (Hammond-Kosack et al., 1994a), a second source of IF(Avr9$^+$) was recovered. IF obtained from untransformed tobacco plants grown under the identical greenhouse conditions served as the comparable IF(Avr9$^-$) control.

The interveinal regions of fully expanded leaves were injected with a serial dilution of the different IFs to determine the dilution end point at which the normal gray necrotic reaction conditioned by the *Cf-9* resistance gene was still detectable (Hammond-Kosack and Jones, 1994). The two IF(Avr9$^-$) preparations were used to ascertain whether the specificity of the *Cf-9*/*Avr9*–mediated response was maintained in other plant species.

Histological Analyses

To determine host cell viability, we stained leaf samples with trypan blue by the method of Keogh et al. (1980). Stomatal density was also measured, using the same staining procedure, from leaf discs removed from the lamina section opposite the site of IF injection. To examine the generation of reactive oxygen species (ROS), leaf discs were cut at various time intervals after IF injection and stained with nitro blue tetrazolium (NBT), as previously described (May et al., 1996).

ACKNOWLEDGMENTS

We thank Pierre De Wit for making available *C. fulvum* race 5.9 and Sarah Perkins and Jonathan Darby for their excellent horticultural assistance. We thank Colwyn Thomas and David Jones for helpful discussion. This research was supported by the Gatsby Charitable Foundation.

Received March 2, 1998; accepted June 12, 1998.

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