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Identification of Two Genes Required in Tomato for Full *Cf-9*-Dependent Resistance to *Cladosporium fulvum*

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Mutagenesis was used to identify and characterize plant genes required for fungal disease resistance gene function in tomato. Seed of a stock homozygous for the *Cf-9* gene for resistance to *Cladosporium fulvum* were treated with ethyl methanesulfonate, and 568 M_2 families were screened for mutations to *C. fulvum* sensitivity. Eight mutants with reduced resistance were isolated. Four mutations, all of which mapped to the *Cf-9* gene, lost both resistance and response to the race-specific AVR9 elicitor. The other four mutations partially lost resistance and response to the AVR9 elicitor. Cytological analysis revealed that a unique host cell staining pattern accompanied the reduced-resistance phenotype in three mutants. Two of the mutants with reduced resistance mapped to *Cf-9*, and two mapped to two distinct loci designated *Rcr-1* and *Rcr-2* (Required for *Cladosporium* resistance) that are unlinked to *Cf-9*.

INTRODUCTION

A key objective in plant pathology is to understand how plant disease resistance (*R*) genes control the ingress of pathogens. Plant breeders have used *R* genes to increase varietal resistance to fungal, bacterial, viral, and nematode pathogens, even though their mode of action is unknown. *R* genes usually confer resistance to specific pathogen races, and pathogen variation can overcome them (Flor, 1946). These naturally occurring variations in the pathogen population are usually caused by recessive mutations, and this led Flor (1946) to propose the "gene-for-gene" hypothesis, which holds that for *R* genes to function, there must be corresponding avirulence (*Avr*) genes in the pathogen.

A better understanding of how *R* genes work might lead to the capacity to engineer more effective and stable resistance in crop plants. It would be desirable to understand all the gene functions required to express effective *R* gene–dependent resistance. A genetic approach to this problem is to mutate an *R* gene–containing line and characterize the mutants recovered with increased disease sensitivity. By identifying the ways in which a resistance response becomes defective, a dissection of the genetic requirements for the wild-type response is permitted.

Surprisingly, this mutagenic strategy has been applied infrequently to the study of specific *R* gene action (Pryor and Ellis, 1993). McIntosh (1977) induced mutations chemically in a wheat line containing three linked *R* genes, each conferring resistance to a different fungal species. The data indicate the rate of mutation to disease sensitivity was high, between 0.1 and 1.9% of M_1 spikes, but differed for each *R* gene. It was also noted that many mutants of one *R* gene exhibited an intermediate level of resistance, whereas mutations involving another appeared fully susceptible. In another report, a high frequency (0.24% of M_1 plants) of mutants that had lost resistance were obtained from a barley line containing the mildew resistance gene *Ml-a*$_{12}$ (Torp and Jorgensen, 1986; Jorgensen, 1988). Twenty-two of the 25 mutations recovered mapped to the *Ml-a*$_{12}$ locus, and these novel alleles resulted in a spectrum of modifications to the resistance phenotype. The other three mutations, two recessive and one semidominant, were inherited independently of *Ml-a*$_{12}$ and appeared to suppress its action. Further analysis of the effects of one of these suppressor genes (M_{100}) on other barley mildew *R* genes clearly demonstrates that some, but not all, were weakened by the presence of this recessive gene (J. Jorgensen, unpublished results).

The interaction between tomato and the obligate biotrophic fungal pathogen *Cladosporium fulvum* is well characterized genetically, cytologically, and biochemically. The dominant resistance genes (*Cf* genes) in the host are presumed to encode products that detect products arising from the action of dominant *Avr* genes in the pathogen (Day, 1956). The extracellular mode of hyphal colonization has allowed the isolation of the primary products of these avirulence genes from intercellular fluids (IFs) retrieved from infected susceptible leaves (de Wit and Spikman, 1982). A 28–amino acid peptide, which has necrosis-inducing ability only on tomato genotypes carrying the *Cf-9* resistance gene, has been purified from IF (Schottens-Toma and de Wit, 1988). Amino acid sequence data permitted the subsequent isolation of both cDNA and genomic clones encoding the fungal peptide, and the genomic sequence was used to transform a race 9 (normally virulent on *Cf-9*–containing plants) to an avirulent race (Van Kan et al., 1991).

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The genetic locations of several *Cf* resistance genes are known (Jones et al., 1993). The *Cf-1*, *Cf-4*, and *Cf-9* genes are allelic, or very closely linked to each other on the short arm of chromosome 1 (Kerr and Bailey, 1964; Jones et al., 1993). Detailed cytological information of fungal ontogeny in compatible and incompatible interactions with different *Cf* genes is available (Lazarovits and Higgins, 1976; de Wit, 1977; Hammond-Kosack and Jones, 1994). Recent studies have shown that the *Cf* genes are incompletely dominant and that the resistance phenotypes of allelic or closely linked *Cf* genes are similar (Hammond-Kosack and Jones, 1994). Accumulating biochemical and molecular data indicate that incompatibility is associated with the synthesis of reactive oxygen intermediates (O_2^-, H_2O_2, and $OH^·$), ethylene, increases in lipoxygenase activity, and the accumulation of a number of pathogenesis-related proteins, including P14, P67, and various glucanases and chitinases (de Wit et al., 1986; Joosten and de Wit, 1989; Peever and Higgins, 1989; Vera-Estrella et al., 1992; K.E. Hammond-Kosack, unpublished results). Callose and lignin deposition and rapid host cell death at the infection site have sometimes been reported, but fungal arrest is possible in the absence of these responses (Lazarovits and Higgins, 1976; de Wit, 1977; Hammond-Kosack and Jones, 1994). Clearly, many of the candidate molecules for plant signaling and plant defense are induced in *Cf*-dependent resistance responses. However, their causal involvement in resistance is unproven but may be tested by mutational analysis. Tomato is also attractive for this type of study because many of these putative components of the resistance response have already been isolated and characterized from its genome or from the related solanaceous species tobacco, potato, and petunia.

In this paper, we report the isolation of tomato mutants with reduced resistance from a line of tomato containing *Cf-9*. Mutants were identified by using a *C. fulvum* race that expresses the *Escherichia coli uidA* gene that encodes β-glucuronidase (GUS) (Oliver et al., 1993). The extracellular mode of *C. fulvum* hyphal growth permitted GUS activity assays to be performed directly on infected plant material. Sensitive mutants had higher GUS activity due to the raised levels of fungal biomass in their apoplasts. The genetic relationships of the mutants to the *Cf-9* gene and to each other were defined. The infection phenotypes each mutant confers were characterized microscopically to see how the defense response was altered.

RESULTS

Frequency of Visible Mutations

To assess the general effectiveness of the ethyl methanesulfonate (EMS) seed treatment in inducing mutations and to compare the relative frequency of occurrence of mutants with reduced resistance to the overall frequency of mutants, the M_2 families were scored for visible mutations on the cotyledons and hypocotyls prior to inoculation with *C. fulvum*, and leaf mutations were identified while screening for fungal biomass. In Table 1, a list of the categories of visible mutant phenotypes identified during the first 28 days of seedling growth and their overall frequency are presented. The distribution of ratios of wild type to mutants identified in the M_2 families for each class of mutants is also indicated. Overall, the frequency of visible mutations was high with 34% of M_2 families exhibiting at least one aberrant phenotype. An additional 26 M_1 plants (4.4%), which were planted for M_2 seed production, did not yield seed and 19 of the viable M_1 plants (3.2%) were chimeric. The chimeric plants had a wild-type main stem and mutant chlorotic and/or necrotic lateral shoots. Thus, the generated M_2 population appeared to be suitable for screening for reduced-resistance mutants.

Table 1. Frequency of Visible Mutant Phenotypes Found in 568 M_2 Families

Phenotype	No. of M_2 Families Segregating for the Mutation[a]	Mean Frequency of Occurrence in Each M_2 Family Segregating for the Mutation
Low germination (<75%)	97 (17.0)	0.258
Dwarf	57 (10.0)	0.263
Completely chlorotic cotyledons	34 (6.0)	0.247
Altered cotyledon morphology	28 (4.9)	0.239
Triple or quadruple cotyledons	17 (3.0)	0.130
Albino	15 (2.6)	0.198
Anthocyanin absent from hypocotyl	11 (1.9)	0.151
Autonecrotic	3 (0.5)	0.270
High anthocyanin pigmentation	3 (0.5)	0.292
Altered leaf morphology	2 (0.35)	0.325

[a] Numbers within parentheses indicate percent.

An interesting class of mutation was found in three M_2 families that segregated for necrotic individuals. These mutants resembled disease lesion mimic mutants that spontaneously develop necrotic lesions even when grown in sterile culture (Walbot et al., 1983). Similar mutations have been recovered in other species when EMS but not diepoxybutane or x rays were used as the mutagenic agent (Walbot et al., 1983). A detailed analysis of these tomato necrotic mutants, especially with regard to possible roles in disease resistance, will be presented elsewhere (K.E. Hammond-Kosack, unpublished results).

Frequency of Mutants with Reduced Resistance

Twenty-five M_2 seedlings from each of 568 M_1 plants were screened for their response to *C. fulvum*. The total number of seedlings screened was ~14,200. Screening was performed

at the cotyledon stage, and a strain of *C. fulvum* that constitutively expresses high levels of GUS activity in vitro and in the plant (Roberts et al., 1989; Oliver et al., 1993) was used. M_2 families containing individuals with reduced resistance were distinguishable 11 to 12 days after inoculation due to the high levels of GUS activity associated with increasing fungal biomass. Screening involved 4-methylumbelliferyl β-D-glucuronide (MUG) assays performed directly on infected plant material. The assays identified a family and then the individuals within it responsible for the elevated GUS activity. The results are shown in Figures 1 and 2. By using this approach, eight M_2 families (1.3%) that segregated for progeny supporting increased fungal biomass were identified. The details of these eight M_2 families are given in Table 2. In a separate experiment to test the stability of the *Cf-9* gene, no susceptible individuals were identified in 15,000 Cf9 × Cf0 F_1 plants screened for fungal sporulation (M. Dixon, unpublished results). Two classes of mutants were identified: those that permitted *C. fulvum* sporulation (M2, M140, M339, and M466) and those that allowed increased vegetative mycelial growth but no sporulation (M21, M105, M164, and M525). The mutants were present

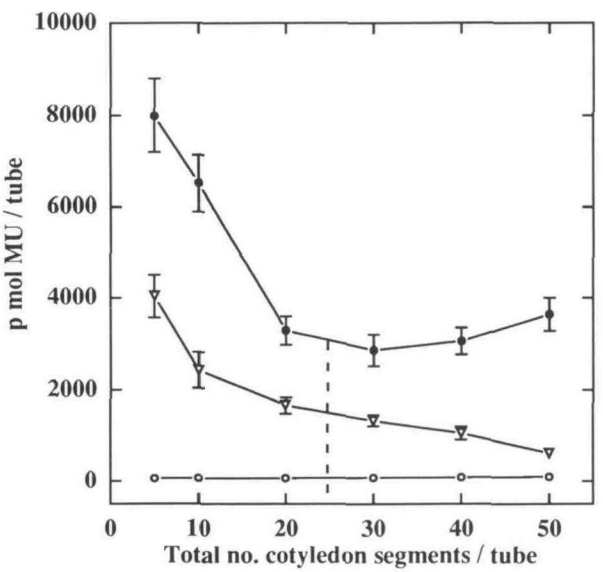

Figure 1. Calibration of a MUG Assay to Identify M_2 Families Containing Mutant Individuals with Reduced Resistance to *C. fulvum*.

MU levels obtained by MUG assays of different numbers of pooled cotyledon segments infected with *C. fulvum* race 4 *GUS* are shown. The segments were cut 11 days after whole-plant inoculations. ○, Cf9 segments alone; ▽, Cf9 segments spiked with one Cf3 segment; ●, Cf9 segments with one Cf0 segment. The resistance conferred by the *Cf-9* gene permitted only minimal vegetative hyphal growth, whereas the resistance conferred by the *Cf-3* gene allowed extensive vegetative hyphal growth but minimal sporulation. Cf0 is fully susceptible to *C. fulvum*. Vertical bars represent standard deviations of the means. The vertical dashed line indicates the population size used to screen the M_2 families for mutants with reduced resistance.

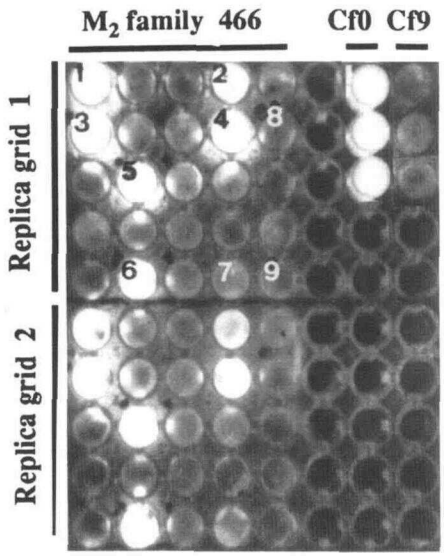

Figure 2. MUG Assays to Identify Individual Plants within the M_2 Family M466 with Increased Fungal Biomass.

Pooled M_2 family M466 had a MU level 30 times greater than the Cf9 control. Two cotyledon segments were taken from each M2 plant 12 days after inoculation, and each was placed in a single microtiter well containing MUG buffer. Duplicate 5 × 5 sample layouts were established that corresponded to the seedling layout in the pot. Control segments of infected Cf0 and Cf9 plants were also included. MUG assays were performed as described in Methods. Mutant plants with increased fungal biomass were detectable when the microtiter plate was viewed on an UV transilluminator. The numbers 1 to 9 indicate the plants selected for rescreening at the two fully expanded leaf stage.

in each family at a frequency of between 0.125 and 0.5. This result is largely consistent with the concept of one genetically effective cell in tomato for the third fruit truss and confirmed the earlier conclusion of Redei (1975). The stable inheritance of the mutant phenotypes listed in Table 2 has been demonstrated to at least the M_4 generation for each mutant family. The authenticity of the four fully susceptible mutants was verified by DNA gel blot analysis using the probes CP46, TG301, and CT197 (Balint-Kurti et al., 1994) that are closely linked to the *Cf-9* gene and reveal restriction fragment length polymorphisms (RFLPs) between Cf0 and Cf9 lines. The RFLPs for mutants M2, M140, M339, and M466 were identical to those obtained for the Cf9 line when each probe was used (data not shown).

Genetic Analysis of Mutants

To determine the genetic basis of the reduced-resistance phenotype in the eight mutant lines, reciprocal crosses of the mutants to Cf0 and Cf9 lines and to each other were performed. When crossed to Cf0, mutants at the *Cf-9* locus would give F_1 progeny that show susceptibility or reduced-resistance. Obtaining

Table 2. Quantification of Fungal Biomass with *C. fulvum* Race 4 *GUS* of Eight Reduced-Resistance Mutants and Wild-Type Cf0 and Cf9 Plants and the Frequency of the Mutation in Each M_2 Family

Mutant and Wild-Type Tomato Lines	MU Values		Sporulation Onset[b]	Frequency of Mutant in M_2 Family[c]	GECN[d]
	Absolute ± SD[a]	Normalized to Cf9			
M2	14,314 ± 2,107	80.0	10.3 ± 0.3	7/25	1.0
M140	13,782 ± 2,035	77.0	10.1 ± 0.2	3/24	2.0
M339	15,479 ± 2,314	86.5	10.7 ± 0.4	3/11	0.9
M466	13,107 ± 2,182	73.2	10.6 ± 0.3	6/25	1.0
M21	981 ± 136	5.5	ND	6/12	0.5
M105	2,508 ± 394	14.0	ND	6/24	1.0
M164	1,523 ± 227	8.5	ND	4/20	1.3
M525	678 ± 91	3.8	ND	6/25	1.0
Cf0	15,978 ± 2,211	89.3	10.2 ± 0.3		
Cf9	179 ± 36	1.0	ND		

[a] Values are given in picomoles of MU per milligram of protein per minute. Fungal biomass was determined 14 days after leaf inoculation of plants by MUG assays. The mean wild-type sibling value was 189 ± 31. Data are from M_2 and M_3 plants.
[b] Days after inoculation when conidiophores were first detected emerging through stomata in the lower cotyledon surface. Assessments were based on microscope observation of X-gluc–stained samples from a minimum of 24 plants for each genotype. ND indicates none detected.
[c] Frequency of segregation in M_2 families; numerator, number of segregating mutants; denominator, total number in a family.
[d] Genetically effective cell number estimated according to Redei (1975): GECN = t/4a, where t = total number of plants in a specific M_2 family, and a = total number of homozygous mutants in a specific M_2 family. The low value for M21 was a result of the segregation in repulsion of a seedling lethal phenotype.

wild-type resistant F_1 progeny would indicate a mutation at another locus. Likewise in the intermutant crosses, obtaining susceptible or reduced-resistance F_1 progeny would indicate that the two mutations were at the same locus, whereas wild-type resistant F_1 progeny would indicate that the two mutations were at two distinct loci. The segregation data obtained from reciprocal crosses were identical; therefore, the results were pooled. Six of the mutants behaved alike genetically, but mutants M21 and M105 were distinct. Table 3 shows the responses of the F_1 progeny from crosses between the mutants and Cf0 and Cf9 lines to *C. fulvum* infection. Table 4 shows the corresponding responses to IF. In the crosses to Cf0, all F_1 plants

Table 3. Quantification of Fungal Biomass with *C. fulvum* Race 4 *GUS* in the Eight Reduced-Resistance Mutants and Their F_1 Progeny Derived from Crosses with Cf0 and Cf9

Mutant and Wild-Type Tomato Lines	Gene Mutated	MU Values ± SD[a]		
		M_4	M_3 × Cf0	M_3 × Cf9
M2	*Cf-9*	13,721 ± 2,108	12,960 ± 1,821	230 ± 28
M140	*Cf-9*	15,320 ± 2,373	13,108 ± 2,178	194 ± 27
M339	*Cf-9*	16,410 ± 2,886	14,744 ± 2,227	230 ± 25
M466	*Cf-9*	15,987 ± 2,042	14,088 ± 2,070	187 ± 33
M21	*Rcr-1*	1,014 ± 142	231 ± 34	241 ± 36
M105	*Rcr-2*	2,618 ± 320	191 ± 27	178 ± 22
M164	*Cf-9*	1,473 ± 281	2,731 ± 424	221 ± 29
M525	*Cf-9*	674 ± 107	834 ± 122	237 ± 24
Cf0		14,528 ± 2,277		
Cf9		182 ± 33		
Cf0 × Cf9		246 ± 34		

[a] Cotyledons on day 14 after inoculation were assessed for fungal biomass by MUG assays. Assessments were based on a minimum of 36 plants for each genotype.

Table 4. Cotyledon Responses to Race-Specific-Elicitor (IF) of Eight Reduced-Resistance Mutants and Their F_1 Progeny Derived from Crosses with Cf0 and Cf9

Mutant and Wild-Type Tomato Lines	Gene Mutated	Response to IF[a]		
		M_4	M_3 × Cf0	M_3 × Cf9
M2	*Cf-9*	–	–	32
M140	*Cf-9*	–	–	32
M339	*Cf-9*	–	–	32
M466	*Cf-9*	–	–	32
M21	*Rcr-1*	16	32	64
M105	*Rcr-2*	8	32	64
M164	*Cf-9*	16	8	64
M525	*Cf-9*	32	16	64
Cf0		–		
Cf9		64		
Cf0 × Cf9		32		

[a] The gray necrosis-inducing activity of the IF preparation is expressed as the reciprocal of the dilution end point that still induced the response on cotyledons. All assessments were based on a minimum of 16 plants for each genotype. –, No response to IF.

derived from the four fully susceptible mutants displayed no resistance. F_1 plants from two of the partially susceptible mutants, M164 and M525, permitted additional *C. fulvum* growth and exhibited a weaker response to IF challenge than the corresponding mutant lines.

The resistance gene *Cf-9* exhibits incomplete dominance, i.e., infections are confined more efficiently when the gene is homozygous rather than heterozygous (Hammond-Kosack and Jones, 1994). This property is discernible only upon microscopic observation of infection sites and not by quantitative MUG assays after challenge with race 4 *GUS* (Oliver et al., 1993) because extensive *C. fulvum* hyphal growth on both cotyledon and leaf surfaces prior to penetration produces a moderate background level of GUS activity in all plant samples. The small rise in GUS activity caused by the increased hyphal growth inside the plant in a *Cf-9* heterozygous line is thereby masked. However, when challenged with IF, the *Cf* homozygotes can respond to give a macroscopically visible gray necrotic reaction at a twofold lower titer of IF than that of the *Cf* heterozygote (Hammond-Kosack and Jones, 1994). In the crosses to Cf9, the four fully susceptible mutants conditioned a resistance response equivalent to that of a *Cf-9* heterozygous resistance phenotype, and the two partially susceptible mutants, M164 and M525, conditioned a resistance response equivalent to that of an intermediate between a wild-type *Cf-9* homozygote and a *Cf-9* heterozygote. Thus, these mutations were not dominant to *Cf-9*. F_2 populations derived from the cross of Cf0 to these six mutants did not segregate homozygous wild-type *Cf-9* resistance phenotypes (data not shown). Collectively, the data indicated that mutants M2, M140, M339, M466, M164, and M525 are all in the *Cf-9* gene.

In Table 5, the IF responses and infection types of the F_1 progeny from the intermutant crosses are shown. These data confirmed the allelism of the six *Cf-9* mutants. Only fully susceptible, non-IF-responsive F_1 progeny were obtained from

Table 5. Infection Types with *C. fulvum* Race 4 *GUS* and IF Responses of Eight Mutants with Reduced Resistance in Diallelic Crosses

Crosses between	MU Value[a]	Response to IF[b]
Fully susceptible mutants that map at *Cf-9*		
M2 × M140	14,217 ± 2,135	–
M2 × M339	13,812 ± 1,975	–
M2 × M466	16,317 ± 2,274	–
M140 × M339	16,310 ± 2,178	–
M140 × M466	15,370 ± 1,891	–
M339 × M466	14,827 ± 2,063	–
Reduced resistance and fully susceptible mutants that map at *Cf-9*		
M164 × M525	904 ± 124	16–32
M164 × M2	2,631 ± 373	8
M164 × M140	1,973 ± 319	8
M164 × M339	2,131 ± 294	8
M164 × M466	2,754 ± 301	8
M525 × M2	797 ± 115	16
M525 × M140	831 ± 141	16
M525 × M339	899 ± 107	16
M525 × M466	913 ± 139	16
Mutations at *Rcr-1* and *Cf-9*		
M21 × M2	251 ± 32	32
M21 × M140	214 ± 33	32
M21 × M339	174 ± 29	32
M21 × M466	261 ± 34	32
M21 × M164	227 ± 28	64
M21 × M525	208 ± 31	64
Mutations at *Rcr-2* and *Cf-9*		
M105 × M2	242 ± 37	32
M105 × M140	189 ± 28	32
M105 × M339	231 ± 28	32
M105 × M466	198 ± 30	32
M105 × M164	178 ± 29	64
M105 × M525	243 ± 28	64
Mutations at *Rcr-1* and *Rcr-2*		
M21 × M105	252 ± 33	64

[a] Values are given in picomoles of MU per milligram of protein per minute. Fungal biomass was assessed by MUG assays 14 days after cotyledon inoculations. Assessments were based on a minimum of 24 plants for each genotype.

[b] Reciprocal value of the dilution titer used (see footnote, Table 4). Assessments were based on a minimum of 16 plants for each genotype. –, No response to IF.

crosses between the fully susceptible mutations M2, M140, M339, and M466, and when these four mutants were crossed to either of the partially susceptible mutants M164 or M525, the phenotypes were equivalent to those of the M164 × Cf0 and M525 × Cf0 F_1 progeny. The intermediate phenotype of the M164 × M525 F_1 progeny to IF challenge also suggested that these mutant genes are allelic to *Cf-9*. The six mutant alleles of *Cf-9* have been designated *Cf-9*^{M2}, *Cf-9*^{M140}, *Cf-9*^{M339}, *Cf-9*^{M466}, *Cf-9*^{M164}, and *Cf-9*^{M525}.

The F_1 plants from the crosses between the other two partially susceptible mutants, M21 and M105, and Cf0 exhibited wild-type *Cf-9* heterozygous resistance responses to both pathogen and IF challenges (Tables 3 and 4). The M21 × Cf9 and M105 × Cf9 F_1 progeny gave wild-type *Cf-9* homozygous responses to both challenges. When intercrossed, the M21 × M105 F_1 progeny again exhibited a wild-type *Cf-9* homozygous resistance phenotype (Table 5). Collectively, these data suggested that the two mutations were not at the *Cf-9* locus but were at two other independent loci. The F_2 populations derived from the crosses M21 × Cf0 and M105 × Cf0 are shown in Table 6. The progeny segregated in a ratio of 3:6:3:4 for seedlings with infection types equivalent to the *Cf-9* homozygote, *Cf-9* heterozygote, mutant, and fully susceptible phenotype, respectively (Table 6). If the mutated locus was linked to *Cf-9*, the expected segregation ratio for these phenotypes would have been 0:2:1:1. Clearly, the key indicator of linkage in this analysis is the frequency of *Cf-9* homozygotes in the F_2 population. Microscopically, incompatible infections conferred by the homozygous *Cf-9* gene are easily distinguishable from those conferred by the *Cf-9* heterozygote (Hammond-Kosack and Jones, 1994). Therefore, obtaining a 3-to-13 frequency of *Cf-9* homozygotes in the F_2 progeny of the crosses M21 × Cf0 and M105 × Cf0 indicated that both mutant loci are unlinked to *Cf-9*.

The F_2 population derived from M21 × M105 segregated in a ratio of 9:3:4 (wild type:M21:M105 phenotypes). Thus, the double mutant phenotype, expected in one sixteenth of the F_2 progeny, has a phenotype indistinguishable from that of the M105 mutant. If the mutations M21 and M105 were tightly linked to each other, a 2:1:1 segregation ratio would be expected in the F_2 population. As the observed data do not fit this ratio ($\chi^2 = 9.139$, $P < 0.02$), we concluded that the two mutant loci were inherited independently of each other. Since the *Cf-9* gene is incompletely dominant, it is likely that both M21 and M105 are homozygous for the *Cf-9* gene; in addition, both have separate recessive mutant genes that were inherited independently of *Cf-9* as well as each other. Both recessive genes modified the phenotypic expression of *Cf-9* and two of its mutant alleles, i.e., *Cf-9*^{M164} and *Cf-9*^{M525} (Table 5).

The genes that were mutagenized in lines M21 and M105 were designated *Rcr-1* and *Rcr-2* (Required for *Cladosporium* resistance), and the corresponding plant lines containing these mutant genes were called Rcr1 and Rcr2, respectively.

Phenotypes of Mutants with Reduced Resistance

The accumulation of *C. fulvum* biomass and the onset of sporulation in the four *Cf-9* mutants M2, M140, M339, and M466 appeared identical to that observed on the Cf0 line, which contains no known *Cf* resistance genes, as shown in Table 2 and Figure 3A. However, 17 days after inoculation, i.e., 3 days after sporulation commenced, considerably more chlorosis and necrosis were consistently evident for three of these lines (all except M339) than for Cf0 (Figure 3B). Identical results were obtained whether race 0 or race 4 *GUS* was used.

On detailed microscopic and histological analysis of the infection time courses, all four mutants were distinct from Cf0. Besides the X-gluc stain, lactophenol-trypan blue was used to assess both host cell viability and hyphal growth (Keogh et al., 1980). Early and often extensive occlusion of vascular tissue with brown phenolic material and the death of adjoining mesophyll cells accompanied sporulation in each mutant but was absent in Cf0, as shown in Figure 4. These host

Table 6. Infection Types with *C. fulvum* Race 0 of Parents and of F_1 and F_2 Progeny Segregating in Crosses with Mutants Rcr1 and Rcr2

Parent		Infection Type[a]			No. of F_2 of Each Infection Type					F_2 Segregation Analysis	
										Phenotype	
1	2	1	2	F_1	Hom	Het	>Het	S	Σ	Ratios	χ^2 Value
Cf0	Cf9	S	Hom	Het	21	35	0	15	71	1:2:1	1.03 ($P > 0.5$)
Rcr1	Cf0	>Het	S	Het	30	87	34	56	207	3:6:3:4	4.07 ($P > 0.2$)
Rcr1	Cf9	>Het	Hom	Hom	87	37	0	0	124	3:1	1.55 ($P > 0.2$)
Rcr2	Cf0	>Het	S	Het	42	101	38	73	254	3:6:3:4	4.37 ($P > 0.2$)
Rcr2	Cf9	>Het	Hom	Hom	107	30	0	0	137	3:1	0.70 ($P > 0.3$)
Rcr1	Rcr2	>Het	>Het	Hom	108	0	29[b]:49[c]	0	186	9:3:4	1.23 ($P > 0.5$)

[a] Assessed by microscopic observation. Hom, phenotype equivalent to that of *Cf-9* homozygote; Het, phenotype equivalent to that of *Cf-9* heterozygote; >Het, significantly more fungal biomass than the *Cf-9* heterozygote; S, susceptible, equivalent to Cf0.

[b] Rcr1 infection type.

[c] Rcr2 infection type.

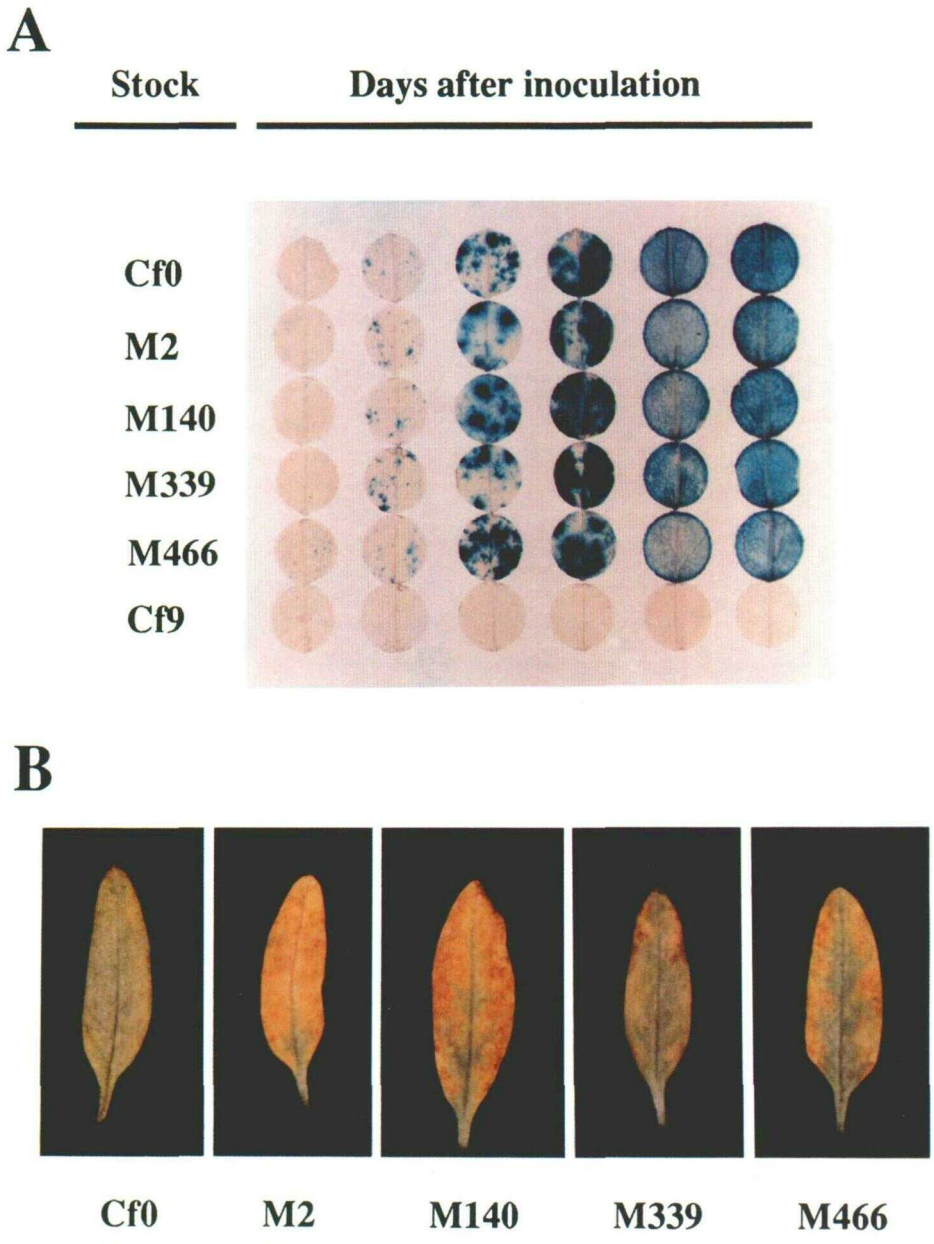

Figure 3. Phenotypes of the Disease-Sensitive Mutants.

(A) Time course of growth of *C. fulvum* race 4 *GUS* in Cf0, Cf9, and the disease-sensitive mutants M2, M140, M339, and M466. Cotyledon discs were excised at various times after plant inoculation and stained with X-gluc. The indigo dye denotes the location of hyphae. The weaker X-gluc staining, evident from day 12 onward in the Cf0 and mutant samples, was due to the detachment of mature conidia containing the indigo dye from samples during the staining procedure.

(B) Macroscopic appearance of cotyledons of Cf0 and the disease-sensitive mutants M2, M140, M339, and M466 17 days after *C. fulvum* inoculation with race 0.

responses were first observed near the lateral margins of the cotyledon and gradually spread inward to the midrib. Changes were first evident in M140 at the onset of conidiophore emergence on day 10, appeared next in M2 (day 12), and last in M466 and M339 (day 14). For M339, these host responses never extended to the midrib. Figures 4A and 4B show the infection phenotypes in Cf0 and M140 in the plane of the vascular tissue, and Figures 4C and 4D show the corresponding infections in the focal plane just above the lower epidermis 14 days after inoculation. When M140 × Cf0 F_1 plants were

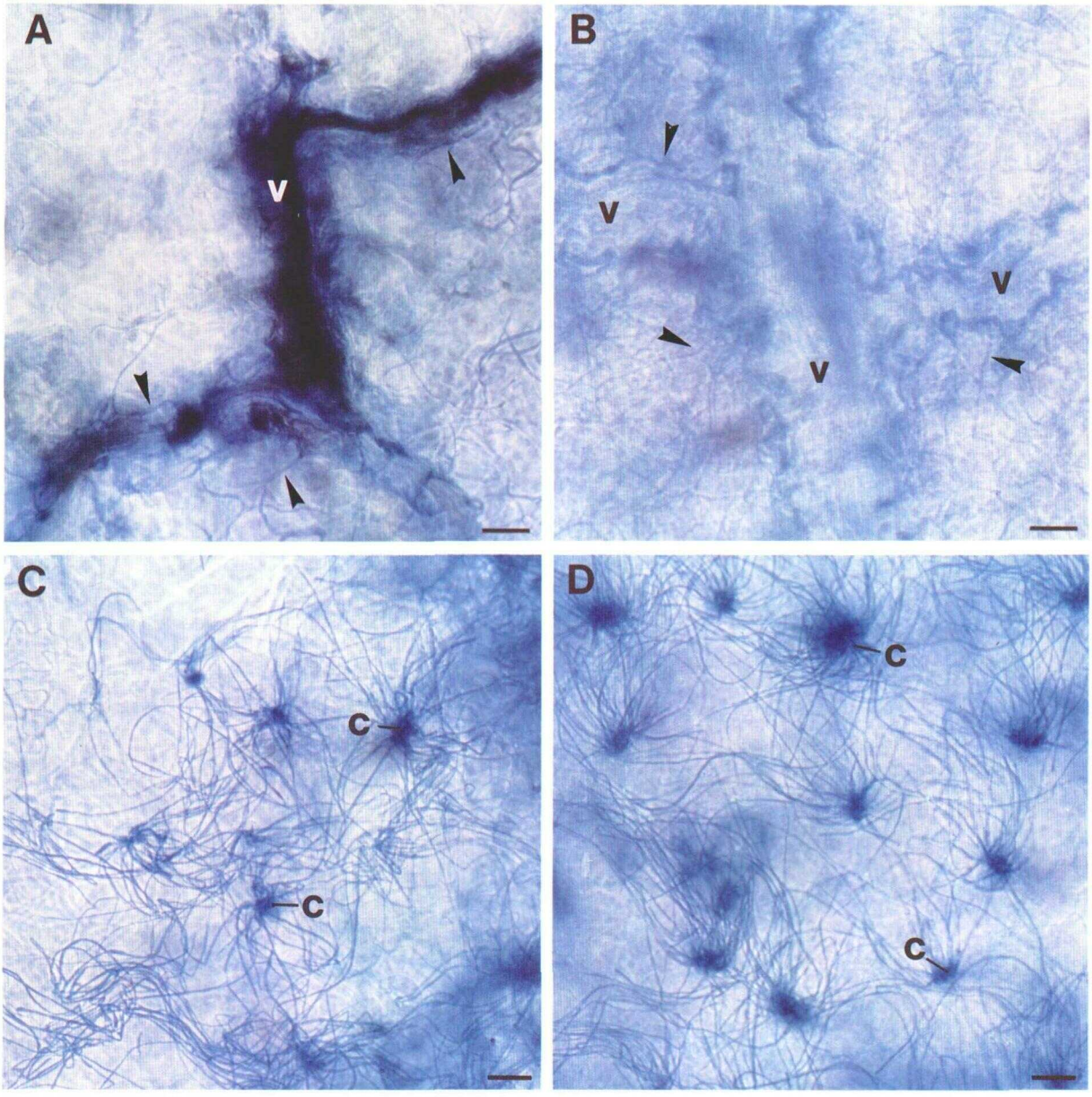

Figure 4. Comparison of *C. fulvum* Infection Types Involving Race 4 *GUS* in Cf0 and the Fully Susceptible Mutant M140.

Photomicrographs were taken of whole-tissue mounts stained with lactophenol-trypan blue.
(A) and **(B)** Accumulation of highly branched hyphae (arrows) around vascular tissue with associated xylem vessel occlusion and dead host cells (v) in an M140 mutant plant in **(A)** but without host cell modification in a Cf0 plant in **(B)** 14 days after inoculation.
(C) and **(D)** Numerous conidiophores (c) emerging from stoma of the lower cotyledon surface of an M140 mutant plant in **(C)** and a Cf0 plant in **(D)** 14 days after inoculation.
Bars = 20 μm.

backcrossed to Cf0 and the BC1 progeny inoculated with *C. fulvum*, the segregation of two distinct susceptible phenotypes was apparent. For 27 plants, sporulation was accompanied by extensive chlorosis and necrosis, whereas for 32 plants only sporulation was evident 17 days after inoculation. These results do not deviate significantly from a 1:1 ratio of wild-type Cf0 to mutant plant (χ^2 = 0.51, $P > 0.4$). DNA gel blot analysis of 12 plants from each category, using the linked probe CP46, confirmed the presence of the *Cf-9*–linked RFLP only in the progeny with the novel phenotype (data not shown).

The four other reduced-resistance mutants Rcr1, Rcr2, M164, and M525 permitted greater amounts of vegetative hyphal growth than that observed on the original Cf9 line. The relative increase in the final lesion diameter compared to those observed in the original Cf9 line, as assessed by microscopic observation, was ×2.8 (±0.31) for M525, ×4.4 (±0.49) for Rcr1, ×5.21 (±0.46) for M164, and ×14.0 (±0.89) for Rcr2, as shown in Figure 5. Each mutant gave a distinct pattern of hyphal growth and accompanying host response. In cotyledons, incompatibility conferred by the wild-type *Cf-9* resistance gene was characterized by the restriction of hyphal growth to within two cell lengths of the penetrated substomatal cavity in the mesophyll layer immediately above the lower epidermis and was accompanied by minimal host cell reactions (Figures 5A and 5B). In Rcr1, hyphae elongated considerably before branching but were again restricted to the mesophyll region immediately above the lower epidermis and induced no visible host responses (Figures 5C and 5D). In M525, although hyphae were sparse and lateral branch elongation was minimal, a few hyphae extended through the lower mesophyll to the veins (Figures 5E and 5F). This hyphal growth was accompanied by the extensive accumulation of the indigo dye product of the X-gluc stain in host cells adjacent to but behind hyphal tips. However, when tissue segments were stained with lactophenol-trypan blue, most host cells either did not accumulate the stain or were only weakly stained, indicating that the cells were alive.

In M164, hyphae colonized the mesophyll layer adjacent to the lower epidermis and also extended to the vascular tissue. Again, host cells adjacent to but behind hyphal tips in the deeper mesophyll layers accumulated the indigo dye but were only weakly stained with trypan blue (Figures 5G, 5H, 5I, and 5J). No host cell reaction was evident around the hyphae, which remained just above the lower epidermis. In Rcr2, abundant hyphae colonized the lower mesophyll, although straight-runner hyphae were not evident. Some hyphae also reached the vascular tissue and then branched frequently and became thickened (Figures 5K and 5L). This growth was always accompanied by the accumulation of the indigo dye by host cells behind the hyphal tip, and at the lesion center host cell death was observed by day 10. From day 14 onward, a few conidiophores emerged through stomata in the lesion center, but these elongated less than 10 μm and conidia never formed. No further expansion of infections on Rcr2 was apparent after day 17, even though some hyphae at the lesion margin were surrounded by apparently normal host cells.

When M_3 plants from each mutant family were challenged with race-specific elicitor peptide preparations (IF), none of the fully susceptible mutants gave a visible response, as shown in Table 4. However, the four partially susceptible mutants gave the characteristic gray necrotic reaction, although this was only observed at an IF titer two- to eightfold higher than on the original Cf9 line.

DISCUSSION

In this study, we showed that mutations could be obtained at the tomato resistance gene *Cf-9* and that two other plant genes are required for full *Cf-9* function. Six different EMS M_2 families segregated reduced-resistance mutants that mapped to the *Cf-9* locus. The mutation frequency (1.3% of M_1 plants) was high and attests to the effectiveness of the mutagenic treatment and to the efficiency of the quantitative assay for fungal biomass used to analyze each M_2 family for individuals with reduced resistance. Before this study, it was conceivable that *Cf-9* was a complex locus composed of several linked resistance specificities. This would have had serious implications for transposon-tagging strategies for *Cf-9* gene isolation. The fact that so many *Cf-9* mutations were recovered is therefore encouraging.

The high frequency of *Cf-9* mutations obtained could indicate that *Cf-9* is a large genetic locus. The six mutations of *Cf-9* recovered were recessive, noncomplementing, and they conditioned different infection types. Mutations that retained some functional activity were epistatic to weaker ones. These findings indicated that the *Cf-9* gene probably has a single open reading frame.

Two types of reduced-resistance mutants were recovered; they were either fully susceptible and nonresponsive to the race-specific elicitor AVR9 or partially susceptible and retaining some necrotic response to AVR9. The four fully susceptible mutations mapped to the *Cf-9* gene. Only two of the four mutations with a partial loss of the efficiency of resistance mapped to *Cf-9.* None of the latter category of mutants would have been identified by the visual scoring of M_2 families for disease because sporulation was absent from each mutant even 28 days after inoculation.

Mutations at *Cf-9*

The fully susceptible infection phenotypes of the mutants M2, M140, M339, and M466 were distinguishable from those in Cf0 because of the increased amount of vascular necrosis accompanying fungal sporulation. Interestingly, each mutant exhibited this phenotype, although the severity of its expression differed (M140 > M2 > M466 > M339). This phenotype could indicate a low residual level of *Cf-9*–like activity in each mutant. Alternatively, the phenotype may indicate that these four mutant lines are more susceptible to the consequences of *C. fulvum* pathogenesis than the Cf0 line, i.e., they exhibit a lower level of disease tolerance.

At the time of sporulation, large amounts of AVR9 peptide and several other compatible specific fungal proteins are recoverable from the leaf apoplast (de Wit et al., 1986; Schottens-Toma and de Wit, 1988). Also, a high density of

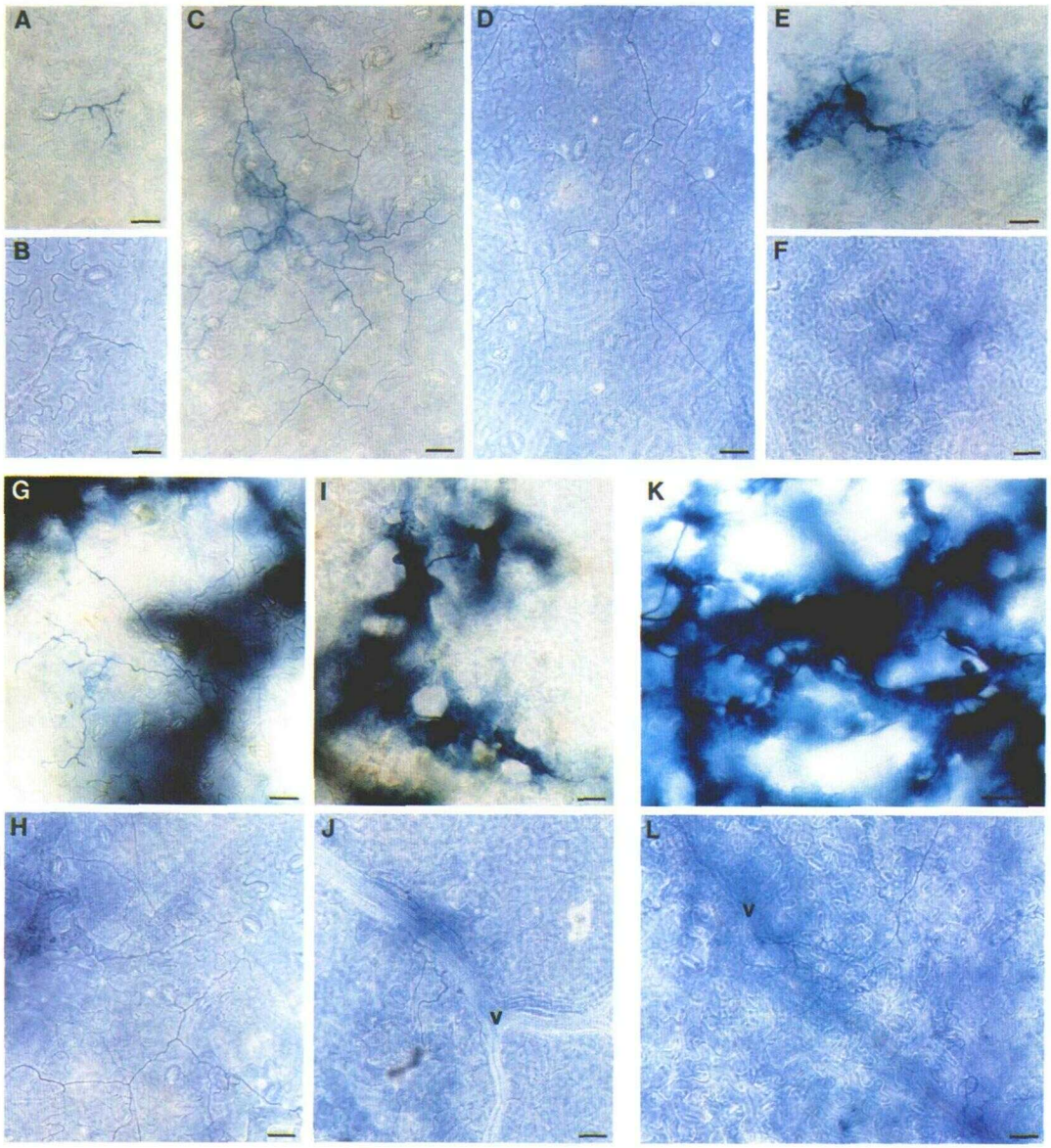

Figure 5. Features of Incompatible *C. fulvum* Interactions in Cf9 and the Four Reduced Disease-Resistant Mutants Rcr1, M525, M164, and Rcr2.

Samples were taken 14 days after inoculation with *C. fulvum* race 4 *GUS*. Photomicrographs are of whole-tissue mounts stained with either X-gluc **(A, C, E, G, H, and K)** or lactophenol-trypan blue **(B, D, F, I, J, and L)**.

(A) and **(B)** Cf9 showing highly branched hyphae with limited elongation; hyphae are situated directly above the lower epidermis. As shown in **(A)** and **(B)**, the X-gluc indigo dye product and trypan blue stain, respectively, were not retained by host cells.

(C) and **(D)** Rcr1 with numerous elongated and branched hyphae restricted to the mesophyll layer directly above the lower epidermis. In **(C)**, some mesophyll cells in the lesion center show weak accumulation of the X-gluc indigo dye product. In **(D)**, cells did not retain the trypan blue stain.

(E) and **(F)** M525 exhibiting limited hyphal growth in the second and third mesophyll layers. In **(E)**, host cells in the lesion center show moderate retention of the indigo dye, whereas in **(F)**, only weak accumulation of trypan blue can be observed.

(G), **(H)**, **(I)**, and **(J)** M164 with numerous hyphae above the lower epidermis and in the deeper mesophyll layers. In **(G)** and **(H)**, the hyphae are situated directly above the lower epidermis; in **(I)** and **(J)**, the hyphae are in the deeper mesophyll layers extending to the vascular tissue (v). The intense accumulation of the indigo dye in **(G)** and **(I)** is only evident in host cells adjacent to hyphae in the deeper mesophyll layers. Weak trypan blue staining in **(H)** and **(J)** is also evident in host cells adjoining colonized vascular tissue.

(K) and **(L)** Rcr2 showing extensive hyphal growth, with hyphae extending throughout the lower mesophyll layer to the vascular tissue (v). In **(K)**, host cells show intense accumulation of the indigo dye, whereas in **(L)** host cells in the lesion center exhibit only weak trypan blue staining. Bars = 20 μm.

hyphae, with a distinct morphological appearance, is associated with vascular bundles (Hammond-Kosack and Jones, 1994). A recent study by Van den Ackerveken et al. (1994) using an *Avr9* promoter::*GUS* gene fusion to analyze the spatial pattern of expression of the *Avr9* gene in the plant has revealed the possibility of an increased abundance of *Avr9* transcript in hyphae surrounding vascular bundles. Conceivably, the function of AVR9 and other extracellular fungal proteins in *C. fulvum* pathogenesis is to promote solute movement through the host cell plasma membrane and thereby enhance the nutrient status of the apoplast where the hyphae habitually reside. Vascular bundle cells, because of their specialized roles in phloem loading and unloading, may be a key target and thus capable of responding to very low levels of agonist. Alternatively, vascular bundle cells may experience a concentration of apoplastic AVR9 peptide that is above the threshold required for the mutant *Cf-9* gene product to trigger a biochemical response.

The increased necrosis associated with vascular tissue at late stages of compatibility in mutants M2, M140, M339, and M466 may indicate the weak action of another *Cf* gene(s). The action of such a gene would hitherto have been masked by the resistance phenotype conferred by the wild-type *Cf-9* gene. Due to the extensive backcrossing strategy used in generating the *Cf-9*–containing near-isogenic line, any other *Cf* gene responsible for this phenotype would have to be linked to *Cf-9*. *Cf-4*, also introgressed into tomato from a different wild *Lycopersicon* species, is known to be closely linked to *Cf-9* (Jones et al., 1993). It is also plausible that other members of a small *Cf-9*–homologous multigene family may reside on the *Cf-9* segment introgressed from *L. pimpinellifolium*. An equivalent severity of necrosis accompanied the sporulation of both *C. fulvum* race 0 and race 4 on the mutants M2, M140, M339, and M466; this indicates that the late necrosis phenotype was not due to the activity of a gene at the locus with the same recognition specificity as *Cf-4*. Interestingly, both McIntosh (1977) and Torp and Jorgensen (1986) also noted that mutations at *R* loci never entirely abolished resistance but instead caused a spectrum of modifications to the resistance phenotype.

The partially reduced-resistance phenotypes of the mutants M164 and M525 not only permitted more hyphal growth but were readily distinguishable from wild-type *Cf-9* infections. In both mutants, the indigo dye product of the GUS stain for the *C. fulvum* transgene accumulated in host cells adjacent to but always behind the hyphal tip. What aspects of the *Cf-9*–dependent defense response might be deficient in these mutants? The wild-type *Cf-9* gene confers a response to AVR9 peptide that includes the rapid (<1 hr) synthesis of reactive oxygen intermediates, lipid peroxidation, and an increase in plasma membrane permeability by 4 hr (Peever and Higgins, 1989; Vera-Estrella et al., 1992). Conceivably, either the temporal relationships between these induced responses have been altered by the action of the mutant *Cf-9* gene product or the delayed onset of the effective resistance permits a build up of AVR9, which later triggers a modified host cell response. The most plausible explanation for the distinct host cell staining pattern is an enhanced uptake of the colorless intermediate product derived from the GUS activity and its subsequent conversion into the indigo dye within the host cells.

Mutations That Do Not Map to *Cf-9*

The two reduced-resistance mutations recovered that did not map to *Cf-9* appeared to be unlinked to *Cf-9* and also to each other. Presumably the wild-type alleles of these genes encode products required for full *Cf-9* function, and when homozygous for the mutant allele at either of these loci, the *Cf-9* gene confers a weaker defense response. These loci were designated *Rcr-1* and *Rcr-2* genes, and the mutant alleles are *rcr-1* and *rcr-2*, respectively. Neither the *rcr-1* mutation (M21) nor *rcr-2* (M105) abolished *Cf-9* function completely, and for both loci only a single mutant allele was recovered. Thus, saturation mutagenesis of *Rcr* genes has certainly not been achieved.

The mutants Rcr1 and Rcr2 exhibited both reduced responses to IF and pathogen challenge. Therefore, it appears unlikely that the increased hyphal growth was merely the consequence of the mutation causing a general increase in the plant's susceptibility to *C. fulvum* infection. The reduced IF response indicated that the mutations *rcr-1* and *rcr-2* cause an alteration to a *Cf*-dependent response. The infection phenotype of Rcr2, like those conditioned by mutations *Cf-9*^{M164} and *Cf-9*^{M525}, was associated with an enhanced accumulation of the indigo dye product of the GUS stain in host cells adjacent to hyphae. In Rcr1 the only phenotypic distinction from the wild-type *Cf-9* response was the greater hyphal biomass permitted before containment.

The mutant resistance phenotypes expressed by *Cf-9*^{M164}, *Cf-9*^{M525}, and *rcr-2* were distinct from those conferred by wild-type *Cf* resistance genes *Cf-1*, *Cf-2*, *Cf-3*, *Cf-4*, *Cf-5*, *Cf-6*, *Cf-7*, and *Cf-11* (Hammond-Kosack and Jones, 1994; K.E. Hammond-Kosack, unpublished results). Thus, these three mutations represent a novel type of *Cf*-mediated resistance to *C. fulvum*, and their phenotypes give a clear indication that incompatibility can be modified. Even in these mutants, growth of *C. fulvum* is still inhibited without any hypersensitive response, which confirms the view that host cell death is not required for pathogen containment (Hammond-Kosack and Jones, 1994).

The mutations at *Rcr-1* and *Rcr-2* are of considerable interest and their further characterization should begin to allow the genetic dissection of *Cf-9*–mediated resistance. These mutations have some features in common with those isolated by Jorgensen (1988) in that they do not map at the *R* locus and they weaken the wild-type resistance response. However, unlike Jorgensen's barley mutants, neither *rcr-1* nor *rcr-2* caused a loss of *R* gene function that permitted fungal sporulation. Induced mutations in tomato and Arabidopsis are being more widely used as a tool to better understand *R* genes (Kunkel et al., 1993; Yu et al., 1993). It might also be expected that these studies will lead to the identification of additional loci

that are required for *R* gene function. To determine whether the defense responses controlled by different linked and unlinked *Cf* genes have similar modes of action to the *Cf-9* gene in the tomato–*C. fulvum* interaction, the *rcr-1* and *rcr-2* mutations have been crossed to lines containing other *Cf* genes.

Once the wild-type *Cf-9* gene has been isolated, the six mutant alleles that map to this genetic locus will be invaluable for the subsequent structure–function analysis of the gene. However, to fully understand how the *Cf-9* resistance gene works, saturation mutagenesis of the tomato genome will be needed to isolate all the loci required for wild-type resistance.

METHODS

Plant and Fungal Material

Near-isogenic lines of the tomato (*Lycopersicon esculentum*) cultivar Moneymaker homozygous for the *Cf-3* or *Cf-9* resistance gene were used. Moneymaker, which carries no known *Cf* resistance gene, was selected as the susceptible parent in the genetic analysis. These plant genotypes are here designated Cf3, Cf9, and Cf0, respectively. A transformed race 4 of *Cladosporium fulvum* (syn. *Fulvia fulva*) expressing the *Escherichia coli uidA* reporter gene encoding β-glucuronidase (GUS) (Oliver et al., 1993) and possessing the avirulence gene *Avr9* was used in the screen for mutants. This race is designated race 4 *GUS*. The analysis of subsequent plant generations also involved a nontransformed race 0 possessing *Avr9*. *C. fulvum* isolates were cultured as described previously (Harling et al., 1988). Both plant cultivars and fungal isolates were obtained from R. Oliver, University of East Anglia, Norwich, Norfolk, UK.

Generation of a Mutagenized Tomato Population and Screening for Reduced-Resistance Individuals in M_2 Families

Six hundred seeds of the homozygous *Cf-9* line were mutagenized by soaking them in a 60-μM aqueous solution of ethyl methanesulfonate (EMS) for 16 hr at 24°C (Koornneef et al., 1987). The third truss of fruit from each M_1 plant was harvested separately to generate the M_2 families for screening. Twenty-five seeds from each M_2 family were sown in 9-cm-diameter pots as a 5 × 5 matrix (with the aid of a perforated plastic grid to ensure correct seed spacing) in Levingtons Universal compost (Fisons, Ipswich, UK). A population size of 25 was chosen in case the fruits were chimeric for mutant sectors or the mutations were linked to gametic or zygotic lethals, thus lowering the mutant transmission frequency. Seedlings were inoculated when the cotyledons were fully expanded and the first true leaf was less than 5 mm in length (~12 days after sowing). To retard seedling elongation, which could become extensive during the infection tests because of the high humidity required for good *C. fulvum* infection (see below), seedlings were treated with the gibberellin inhibitor paclobutrazol (ICI Agrochemicals, Bracknell, UK). Paclobutrazol treatment does not affect *C. fulvum* infections (K.E. Hammond-Kosack, unpublished results). A 50-mL soil drench of 1 mM paclobutrazol was applied to each pot. Two days later, seedlings were inoculated by inverting each pot and submerging the cotyledons into a 5-cm depth of fungal spore suspension (2 × 10^5 conidia per mL of water) contained in a 20 × 13 × 8 cm plastic box.

Inoculated pots of M_2 families were arranged in a 5 × 10 layout on capillary matting and covered with a plastic propagator (152 × 76 × 46 cm) (Melbourne; M.W. Horticultural Suppliers, Kent, UK). Among 47 inoculated M_2 families were placed three control pots: one contained 13 seedlings of Cf9 and 12 seedlings of Cf0; the second contained 24 Cf9 seedlings and one Cf0 seedling; and the third held 25 Cf9 seedlings. For the first 3 days after inoculation, the relative humidity inside the propagators was maintained close to 100% by keeping all air vents closed and was then reduced to ~ 70% by opening some of the vents.

Screening for reduced-resistance individuals was performed 11 days after inoculation, i.e., prior to fungal sporulation. A protocol was devised using race 4 *GUS* to permit the quantification of fungal biomass in the plant. An earlier study had shown that fungal biomass could be quantified directly in individual plant tissue discs (Oliver et al., 1993). Initial experiments indicated this assay could also be used on pools of tissue segments to permit the detection of a single, moderately susceptible seedling in a pool of at least 50 Cf9-resistant seedlings. For the assay, pools of tissue segments were placed in a tube containing 2 mL of 4-methylumbelliferyl β-D-glucuronide (MUG) buffer (Jefferson, 1987). This buffer was composed of 50 mM potassium phosphate buffer, pH 7.0, 5 mM dithiothreitol, 1 mM sodium EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (v/v) Triton X-100, and 1 mM MUG. After vacuum infiltrating the MUG buffer into the samples, the tubes were incubated overnight at 37°C in the dark. Aliquots (2 × 200 μL) of buffer surrounding the tissue samples were then removed from each tube and transferred to microtiter plates; methylumbelliferone (MU) levels were quantified on a fluorometer fitted with a microtiter plate reader (Titertek Fluoroskan II; ICN, Flow Laboratory) (Jefferson, 1987).

In Figure 1, the levels of MU that were detected when a single cotyledon segment (width 5 mm) taken from an infected Cf0 or Cf3 seedling was incubated with different numbers of segments taken from Cf9-infected seedlings (four to 49) are compared to the values obtained with Cf9-infected segments alone. The resistance gene *Cf-3* permits extensive vegetative mycelial growth but minimal sporulation (Lazarovits and Higgins, 1976). These data showed that the presence of a single reduced-resistance seedling within the pool can elevate the obtained MU levels significantly. At a pool size of 25, the values obtained were 39 and 18 times higher for Cf0- and Cf3-containing samples, respectively. Thus, for the screening of each M_2 family a tissue segment (width 5 mm) was cut from just below the tip of one cotyledon of each of 25 seedlings. These tissue segments were placed together in a tube containing 2 mL of MUG buffer. Two control tubes were included; one contained 25 Cf9 tissue segments and the other contained 24 Cf9 tissue segments and one Cf0 tissue segment obtained from the same batch of inoculated plants.

M_2 families that had MU values more than five times that of the Cf9 control were rescreened. Two tissue segments (width 3 mm) were cut from the middle of the cotyledon sampled originally, and each was placed in a single microtiter well containing 150 μL of MUG buffer. Duplicate 5 × 5 sample grids were established, and the layout of the tissue segments relative to the growing positions of the seedlings in the pot was noted. A plastic self-adhesive seal (ICN, Flow Laboratory) was placed over each microtiter plate, and after needle pricks were made above each well, the samples were vacuum infiltrated with buffer and then incubated at 37°C in the dark for 16 hr. Control wells (in triplicate) containing tissue segments from either Cf9 or Cf0 plants (taken from the same inoculation batch) were included on each plate. Plates were analyzed on a UV light box to identify the wells with moderate levels of MU. An example of this analysis is shown in Figure 2. M_2 families in which a consistent pattern of elevated MU was observed on both grids were selected for further screening. The second cotyledon

of each seedling was stained histochemically for GUS activity according to the protocol of Jefferson (1987) using a concentration of 0.5 mg/mL X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cycloammonium salt; Biosynth AG, Zurich, Switzerland) and 1 mM potassium ferricyanide/ferrocyanide as an oxidation catalyst. In addition, six Cf0 and six Cf9 cotyledons from the third control pot in the same inoculation were stained to determine the reference levels of *C. fulvum* growth. A few false positive families were obtained in which the reproducible increases in MU levels could not be attributed to the presence of more fungal hyphae.

Putative mutant individuals with increased levels of fungal biomass and three or four "wild-type" siblings from the same EMS-mutagenized family were potted up individually and moved to a growth room for rescreening when they had two fully expanded leaves. The growth cabinet was maintained at 24°C during the 16-hr light period and 18°C during the 8-hr dark period. Light was supplied by 400-W lamps (Power Star [HQI-T]; Osram Ltd, Middlesex, UK) to give a photon flux density of 600 $\mu E\ m^{-2}\ sec^{-1}$, and the relative humidity was maintained at a constant 70%. The rescreening procedure was identical to that used in the cotyledon screen, except that the plants were spray inoculated (Hammond-Kosack and Jones, 1994). Control Cf0 and Cf9 plants from the first screen were also included, and samples were taken from leaf 2 for fungal biomass determination. Protein levels in samples were estimated by the method of Bradford (1976) using a kit supplied by Bio-Rad Laboratories and bovine serum albumin as the standard. From each M_2 family, three sibling plants that permitted substantially greater hyphal growth than wild-type *Cf-9*–resistant plants were selected for genetic analysis.

Genetic Analyses

Reciprocal crosses between selected EMS reduced resistance mutants Cf0 and Cf9 were made. F_1 and F_2 seedlings were spray inoculated at the cotyledon stage with both race 4 *GUS* and race 0 under the same growth cabinet conditions as described above. In addition, the leaves of F_1 plants were injected with a serial dilution of intercellular fluid (IF) containing *C. fulvum* race-specific elicitors to determine the dilution end point at which the normal gray necrotic reaction conditioned by the *Cf-9* resistance gene was still detectable (Higgins and de Wit, 1985). IF was prepared from a Cf0–race 0 interaction as described by de Wit and Spikman (1982). Chi square analysis was used to test statistically the segregation ratios in the F_2 populations.

To assess the authenticity of the EMS mutants with reduced resistance and to rule out the possibility of non-*Cf-9*–containing contaminant seed among the selected plants, DNA gel blot analyses were performed as described by Jones et al. (1993) using three restriction fragment length polymorphism probes known to be closely linked to the *Cf-9* locus, i.e., TG301, CT197, and CP46 (Gebhardt et al., 1991; Tanksley et al., 1992; Jones et al., 1993; Balint-Kurti et al., 1994).

Additional Cytological Analysis of Mutants Exhibiting Reduced Resistance

Plants from the M_3 and M_4 generations were used to determine the infection type each mutant permitted. Seedlings were inoculated as given above with race 4 *GUS* and race 0, and cotyledon samples were taken 4, 6, 8, 10, 12, 14, 17, and 28 days after inoculation. Besides the X-gluc stain, lactophenol-trypan blue was used to assess both host cell viability and hyphal growth (Keogh et al., 1980). The final extent of hyphal growth in plant tissue was quantified by measuring both the lateral spread of hyphae and the number of mesophyll layers colonized, as previously described (Hammond-Kosack and Jones, 1994). A minimum of 150 infection sites per plant genotype were examined 14 days after inoculation in each experimental replica. Some further samples were taken to identify callose and lignin polymers synthesized by host cells (Lazarovits and Higgins, 1976). A detailed examination of infection types in all the stocks generated by the genetic analysis was also undertaken 14 days after inoculation.

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