Disruption of the Avirulence Gene *avr9* in Two Races of the Tomato Pathogen *Cladosporium fulvum* Causes Virulence on Tomato Genotypes with the Complementary Resistance Gene *Cf9*

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To study the function of the avirulence gene *avr9* of the tomato pathogen *Cladosporium fulvum*, we developed procedures for gene disruption experiments in two different races of the fungus both avirulent on tomato genotypes carrying the resistance gene *Cf9*. For this purpose we selected uridine auxotrophic strains amongst fluoroorotic acid resistant mutants. These mutants were transformed with a plasmid containing the *avr9* genomic region in which the open reading frame was replaced by the *pyrG* gene from *Aspergillus nidulans*. For each of the two races used we selected one transformant in which the entire *avr9* coding sequence was deleted as a result of a gene replacement event. The two transformants were able to successfully infect *Cf9* tomato genotype, unlike their wild-type avr9$^+$ parents, which induced hypersensitive responses on this genotype. We also demonstrated that these two transformants no longer produce the necrosis-inducing elicitor peptide specifically interacting with *Cf9* tomato plants. These results confirm that the cloned *avr9* sequence (and therefore the AVR9 peptide) is fully responsible for avirulence in wild-type avr9$^+$ races of the fungus. These results also indicate that this gene is dispensible for normal vegetative growth and pathogenicity at least in a monocyclic process. These results are discussed in relation to the possible origin of avr9$^-$ strains and the long-standing resistance toward *C. fulvum* offered by the tomato *Cf9* resistance gene.

Additional keywords: gene-for-gene interaction, pathogenicity factor, gene replacement.

The gene-for-gene type of interaction between a pathogen and its host plant postulates the existence of an avirulence gene in the genome of the pathogen whose product interacts with the product of the plant resistance gene to give an incompatible interaction—usually characterized by the development of a hypersensitive response (Flor 1942). This type of interaction can be best demonstrated by performing crosses between avirulent and virulent strains of the pathogen or resistant and susceptible host cultivars and observing the segregation of these characters in the progeny. For pathogenic fungi this is only feasible when sexual or parasexual crosses can easily be performed. This is, for instance, the case for the ascomycete *Magnaporthe grisea* (Valent *et al.* 1991) or the oomycete *Bremia lactucae* (Michelmore *et al.* 1984), pathogens of rice and lettuce, respectively.

For our model species *Cladosporium fulvum* Cooke, a biotrophic fungal pathogen of tomato, no sexual stage has ever been described. Six avirulence genes have been postulated for it based on the differential interactions of wild-type fungal races on tomato cultivars known to possess different resistance genes toward this fungus. The genetic basis of the gene-for-gene interaction between tomato and *C. fulvum* has therefore only been established clearly for the host plant tomato for which the different *Cf* resistance genes have now been positioned on the genetic map (Jones *et al.* 1991; Van der Beek *et al.* 1992). It has also been shown that avirulent races of the fungus produce proteinaceous elicitors that induce a necrotic reaction only when injected into the leaves of the corresponding resistant plant (De Wit and Spikman 1982). Because of the specificities of these elicitors, whose necrosis-inducing activities are reminiscent of the hypersensitive responses observed following the infection of a resistant plant by an avirulent fungal race, it was suggested that they could be the primary products of the avirulence genes.

The necrosis-inducing peptide produced by fungal races avirulent on *Cf9* genotypes (avr9$^+$ strains) was purified and sequenced (Scholtens-Toma and De Wit 1988). The sequence data from this peptide were used to design oligonucleotides that were employed to isolate the cDNA and genomic clones encoding the AVR9 peptide (Van Kan *et al.* 1991 and Van den Ackerveken *et al.* 1992). These authors showed that the genomic coding region is interrupted by one intron and that the primary gene product is a 63-amino-acid-long peptide, from which only the 28

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C-terminal part has been found in infected tomato leaves. Southern hybridization experiments established the presence of this gene in the genomes of all the avr9$^+$ fungal races tested but failed to detect homologous sequences in the genomes of the two races virulent on *Cf9* tomato cultivars tested, suggesting that these isolates could have arisen following a deletion in this genomic region. Karyotype analysis indicated that races 2.4.9.11 and 2.4.5.9.11, both lacking *avr*9, have undergone an estimated deletion of 500 kb from chromosome band 2, suggesting that these virulent races might have lost functional chromosomal DNA in addition to the *avr*9 gene (Talbot *et al.* 1991). Transcripts of the *avr*9 gene could only be detected in susceptible host plant infected by avr9$^+$ races. The genomic sequence could be introduced by transformation and expressed in avr9$^-$ strains, changing them from virulent to avirulent on *Cf9* tomato cultivars. These results clearly demonstrated that the AVR9 peptide is sufficient for triggering the hypersensitive response in combination with the plant resistance gene *Cf9* supporting, therefore, the gene-for-gene hypothesis.

In this paper we present evidence for the disruption of the *avr*9 gene by gene replacement in two different races of the filamentous plant pathogenic fungus *C. fulvum*. These experiments were carried out to definitely confirm the function of this gene, not only as an avirulence gene but also as a potential pathogenicity factor, in avr9$^+$ wild-type isolates. The results obtained give conclusive evidence for the mechanism of avirulence in the tomato-*C. fulvum* interaction and raise interesting questions on the role of *avr*9 in pathogenicity and on the evolution of races lacking this gene.

RESULTS

Isolation and characterization of uridine auxotrophic mutants.

To improve the methodology for the genetic manipulation of *C. fulvum* we tried to isolate mutants that could be used for transformation with either homologous or heterologous genes. For each of the two wild-type races (races 4 and 5, both containing avirulence gene *avr*9), we selected 20 mutants resistant to FOA following UV mutagenesis. Among them, one from race 4 and four from race 5 were uridine auxotrophs. It was shown that in yeast (Boeke *et al.* 1984) the mutants isolated using this method could result from mutations in either the gene encoding the orotidine-5′-phosphate decarboxylase (OMPdecase) or the gene coding for the orotate pyrophosphoribosyl transferase (OPRTase). To decide between these two possibilities, we transformed each of the *C. fulvum* mutants with the cloned *pyr*A gene from *Aspergillus niger* coding for the OMPdecase (Goosen *et al.* 1987). Three of the race 5 mutants and the race 4 one could be efficiently complemented by this heterologous gene and Southern blot analyses indeed confirmed the presence of the *A. niger* gene integrated in their genomes (data not shown). From this result we concluded that these four mutants have a mutation in the structural gene for the OMPdecase and that the fifth one which we could not complement might have a mutation in the gene coding for the OPRTase. Mutants 5.4 from race 5 and 4.2 from race 4 were used as recipient strains for the disruption of the *avr*9 gene.

Construction of a plasmid for the disruption of the *avr*9 gene and transformations.

We designed a plasmid allowing a single step gene replacement at the *avr*9 locus. The *avr*9 coding region present on the lambda clone described by Van den Ackerveken *et al.* (1992) was subcloned as a 6-kb *Eco*RI-*Sal*I fragment into pBluescript (Stratagene). An internal 750-bp *Hin*dIII-*Xho*I fragment containing the whole coding sequence was removed and replaced by the *A. nidulans pyr*G gene present on a 1.5-kb DNA fragment. Figure 1 gives details of the construction of this pCF112 plasmid. The presence of the *pyr*G gene, coding for an OMPdecase, allows the transformation of *C. fulvum* pyr$^-$ mutants to prototrophy. Upstream and downstream of this *pyr*G gene are 2.5 and 2.7 kb of *C. fulvum* DNA sequences that could allow the replacement of the *avr*9 gene following homologous recombination events as illustrated in Figure 1.

pCF112 was linearized with *Eco*RI, which cuts once in the polylinker of pBluescript, and used to transform protoplasts from the pyr$^-$ mutants of races 4 and 5 of *C. fulvum*. Forty-three prototrophic transformants from race 4 and 52 from race 5 were purified before further analyses.

Characterization of avr9$^-$ transformants.

Genomic DNAs extracted from the transformants were initially subjected to dot blot analysis using the 750-bp *avr*9 coding sequence, removed from the transforming plasmid, as the hybridization probe. The DNAs from three transformants (one from race 4 and two from race 5) failed to give any hybridization signal (results not shown), and one such transformant from each race (race 4-A43 and

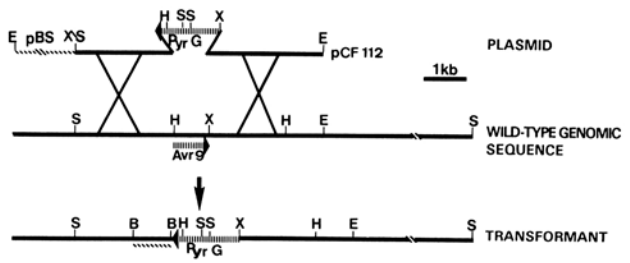

Fig. 1. Construction of the plasmid pCF112 used to disrupt the *avr*9 gene and events leading to the replacement of the gene following transformation. The *avr*9 genomic clone was obtained as a 6-kb *Eco*RI-*Sal*I fragment cloned into the *Eco*RI-*Xho*I sites of pBluescript. A *Hin*dIII site close to the *Eco*RI site was first removed by partial digestion, treatment with Klenow polymerase, and religation of the linear molecule. The plasmid was then reopened at the *Hin*dIII site which covers the second and third codons of the *avr*9 open reading frame. After making the site blunt with Klenow polymerase, the plasmid was digested with *Xho*I to remove the whole of the *avr*9 coding sequence. The *Aspergillus nidulans pyr*G gene was obtained from plasmid pJR15 (Oakley *et al.* 1987) as a 1.5-kb *Nde*I (made blunt with Klenow polymerase)-*Xho*I fragment. Ligation of these two molecules resulted in pCF112, which has the *avr*9 sequences replaced by the *pyr*G gene and which has a single *Eco*RI site used to linearize the plasmid before transformation. Only the restriction sites relevant to this work are indicated: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; and X, *Xho*I. The underlined *Bgl*II fragment was used as the probe in Figs. 2C and 3.

race 5-B51) was subjected to detailed Southern blot analyses along with the DNAs from the wild-type race 5 and from two other transformants (race 4-A11 and race 5-B11) with ectopic integrations of the plasmid. The hybridization to the labeled *avr*9 coding sequence was repeated on *Hin*dIII digested genomic DNAs (Fig. 2A). This probe hybridized to a 2.3-kb DNA fragment of the wild-type race 5 and transformants A11 and B11 but did not hybridize to the DNAs from transformants A43 and B51 (a polymorphism exists between races 4 and 5, seen as a slightly larger hybridizing fragment in the race 4-A11 transformant). ^{32}P-labeled pBluescript sequences only hybridized to the DNAs of transformants A11 and B11 which originated from nonhomologous integrations of the plasmid (Fig. 2B). When an 800-bp *Bgl*II fragment upstream of the *avr*9 gene (Fig. 1) was used to probe *Sal*I digested DNAs, it identified a 10-kb DNA fragment in the genome of the untransformed race 4 and one additional larger fragment in transformants A11 and B11 (Fig. 2C; in this case a DNA polymorphism between the transformants is visible). By contrast, this same probe identified a unique 3-kb fragment in the genomes of tranformants A43 and B51 (Fig. 2C); the size of this last fragment is in agreement with the insertion of the *pyr*G gene, which contains two internal *Sal*I sites.

From these analyses we concluded that transformants A43 and B51 have both arisen from a single step gene replacement at the *avr*9 locus which has been exchanged by the *pyr*G gene present on the transforming pCF112 plasmid. No other integrations of the plasmid at ectopic sites seemed to have occurred in those two transformants. Transformants A11 and B11, containing several ectopic integrations of pCF112, were used as controls to assess the effect of transformation on pathogenicity.

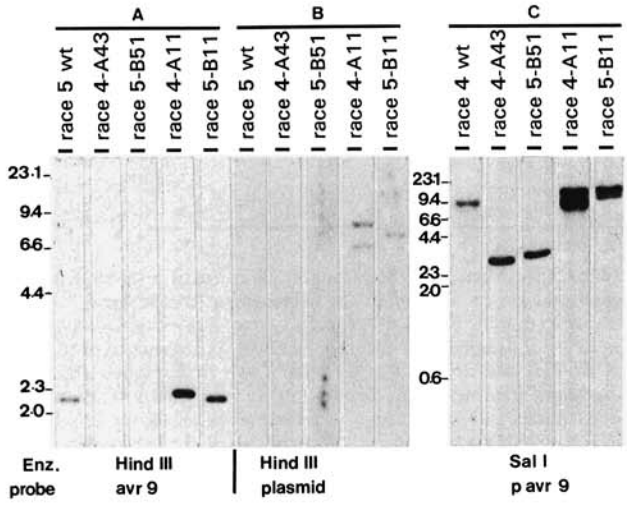

Fig. 2. Southern blot analyses demonstrating that transformants A43 and B51 result from a gene replacement at the *avr*9 locus. The restriction enzymes used to digest the genomic DNAs and the probes used are indicated below the autoradiographs. The hybridization patterns of transformants A43 and B51 are compared to the hybridization patterns given by the wild-type race 5 and two other transformants (A11 and B11) which originated from ectopic integrations of the pCF112 plasmid. Size markers are in kilobases.

Pathogenicity and virulence of the transformants.

Spores from the wild-type races 4 and 5, the corresponding pyr$^-$ mutants, the transformants with a disrupted copy of the *avr9* gene, and the control transformants with ectopic integrations of pCF112 were used to inoculate 2-wk-old tomato seedlings. For each fungal strain, an average of 20 plants from cultivar Moneymaker (MM, *Cf9*$^-$) and its *Cf9* near-isogenic line were used. The symptoms were recorded 2 wk after inoculation. With the exception of the two pyr$^-$ strains, all other strains could infect the MM plants, which were judged by the appearance of a mat of sporulating mycelium on the leaves. Tomato plants carrying resistance gene *Cf9* could only be successfully colonized by transformants A43 and B51, which no longer contain an intact copy of the *avr*9 gene. All other fungal strains failed to infect the *Cf9* plants.

To confirm these observations, DNAs were extracted from similar amounts of infected leaf material, digested with *Sal*I, and subjected to Southern blot analysis using the *Bgl*II-*Bgl*II sequence, present upstream of the *avr*9 gene, as a probe. Hybridizing DNA fragments could easily be detected from infected leaf material 14 days after inoculation (Fig. 3). The presence of a 3-kb fragment clearly identified transformants A43 and B51 (compare with Fig. 2C). The transformants with ectopic integrations contain several copies of the *avr*9 promoter, and the hybridization signal is therefore more intense. No obvious difference could be seen in the intensity of the hybridization signals obtained between the disruption mutants and wild-type *C. fulvum*, indicating that similar amounts of fungal biomass are present in the leaves infected by either the avr9$^+$ strains (MM plants) or the avr9$^-$ transformants (MM and *Cf9* plant).

The ability of the avr$^-$ transformants to infect *Cf9* plants was not simply a consequence of the transformation since transformants A11 and B11 which have ectopic integrations of the pCF112 plasmid retained the host specificities

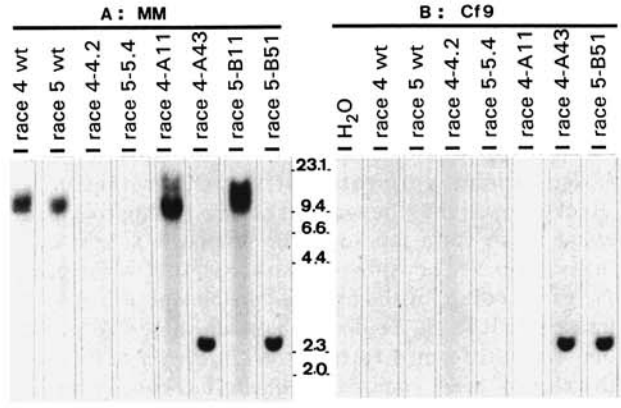

Fig. 3. Southern blot analysis of DNAs extracted from the leaves of infected tomato plants 14 days after inoculation. Genomic DNAs (from both plant and fungal origins) were digested with *Sal*I and the membrane was probed with the *Bgl*II fragment upstream of the *avr*9 gene (as in Fig. 2C). Each lane represents a different fungal strain used to inoculate Moneymaker (MM) and *Cf9* seedlings. The presence of a hybridizing DNA fragment indicates fungal growth and therefore a compatible interaction, the size of the fragment distinguishes between avr9$^+$ strains (10-kb fragment) and avr9$^-$ (*avr*9::*pyr*G) mutants (3-kb fragment).

of the wild-type races 4 and 5, being able to infect MM plants only.

The production of an active elicitor peptide was proven by injecting intercellular fluids collected from the different interactions into the leaves of MM and *Cf9* plants. As summarized in Table 1, a necrotic response could only be detected when intercellular fluids from avr9$^+$/MM interactions were injected into the leaves of *Cf9* plants, while transformants A43 and B51 did not produce any elicitor. Necrotic reactions were observed even when intercellular fluids of avr9$^+$/MM interactions were diluted 10-fold, while undiluted samples of avr9$^-$/MM, *Cf9* interactions failed to induce necrosis.

These results clearly demonstrate that the *avr*9 gene, present in the races 4 and 5 of *C. fulvum*, is the only genetic factor responsible for their inability to infect *Cf9* tomato plants. They also indicate that the AVR9 peptide is the only compound from crude intercellular fluids that induces necrosis in *Cf9* plants.

DISCUSSION

In this paper we demonstrate that a single mutation generated by gene disruption can alter the cultivar specificity of the fungal pathogen *C. fulvum*. In the case of the *avr*9 gene we recovered three transformants that had resulted from a gene replacement out of the 95 we analyzed, which corresponds to a frequency in the range of 1–5%. The data presented confirm that gene disruption by gene replacement is possible in the filamentous plant-pathogenic fungus *C. fulvum* and provides new possibilities for the molecular genetic analysis of avirulence and pathogenicity. Although gene disruption occurs at a low frequency, the use of this method could be extended in the future to assess the function of other *C. fulvum* genes whose products are found in intercellular fluids from infected plants and which are putative pathogenicity factors (Joosten and De Wit 1988; Van den Ackerveken *et al.* 1993). Successful gene replacement has been described for a number of other plant-pathogenic fungi including *Ustilago maydis* (Fotheringham and Holloman 1989; Kronstad *et al.* 1989), *Cochliobolus carbonum* (Scott-Craig *et al.* 1990), *Nectria haematococca* (Stahl and Schäfer 1992), *Magnaporthe grisea* (Sweigard *et al.* 1992), and *Gibberella pulicaris* (Hohn *et al.* 1992).

Table 1. Characteristics of the strains of *Cladosporium fulvum* used in this study[a]

Strain	Description	Cultivars		Production of the AVR9 elicitor
		MM	*Cf9*	
race 5	Wild-type isolate	C	I	+
race 5-5.4	pyr$^-$ mutant induced in race 5 by UV mutagenesis	*	*	
race 5-B51	Race 5-5.4 *avr*9: :*pyr*G insertion mutant	C	C	−
race 5-B11	Race 5-5.4 with the *avr*9: :*pyr*G construct at ectopic sites	C	I	+
race 4	Wild-type isolate	C	I	+
race 4-4.2	pyr$^-$ mutant induced in race 4 by UV mutagenesis	*	*	
race 4-A43	Race 4-4.2 *avr*9: :*pyr*G insertion mutant	C	C	−
race 4-A11	Race 4-4.2 with the *avr*9: :*pyr*G construct at ectopic sites	C	I	+

[a]The outcome of the interaction of different strains of *C. fulvum* with the tomato cultivars Moneymaker (MM, Cf9$^-$) and Cf9$^+$ is indicated; C: compatible interaction (race virulent), I: incompatible interaction (race avirulent). The production of the AVR9 elicitor was assayed by testing, in *Cf9* plants, the necrosis activity of extracellular fluids recovered from infected MM plants two wk after their inoculation. *: nonpathogenic auxotrophic mutants.

In this study, we first selected uracil auxotrophic mutants and showed that the initial selection for fluoroorotic acid resistance can also be applied to *C. fulvum*. The resistant mutants fell into three categories: the prototrophic ones, the auxotrophic ones which could be complemented following transformation by fungal OMPdecase genes, and a third category of auxotrophic mutant which could not be complemented by OMPdecase genes and which may have a mutation in the OPRTase gene. These mutants represent an interesting alternative to the use of bacterial antibiotic resistance genes as selection markers in this species (Oliver *et al.* 1987). However, these pyr$^-$ mutations clearly interfere with pathogenicity on tomato, which was not the case for other auxotrophic mutants selected by Talbot *et al.* (1988).

At the start of this study, we knew from the experiments performed by Van den Ackerveken *et al.* (1992) that the transfer of the *avr*9 sequence to a virulent race of *C. fulvum* was sufficient to make it avirulent on *Cf9* tomato plants. From the results presented in this paper it becomes clear that in the two wild-type races we studied, the *avr*9 gene is indeed the only genetic factor responsible for the induction of an incompatible interaction on tomato *Cf9* plants. This definitively establishes the genetic basis of the gene-for-gene relationship in the case of the *avr*9/*Cf9* interaction and also confirms the direct involvement of the AVR9 peptide in the development of the hypersensitive response. The *avr*9 gene is the only avirulence gene cloned so far for which the processed protein product has been shown to be directly responsible for the induction of the hypersensitive response. This has not been established or does not seem to be the case for most of the bacterial avirulence genes which have been characterized; there is no evidence for their translation products to be excreted and small molecules, generated by the avirulence gene products, seem to act as race-specific signals as in the case of the *avrD* gene from *Pseudomonas syringae* pv. *tomato* (Keen *et al.* 1990). It remains to be proven whether other *C. fulvum* race-specific elicitors described by De Wit and Spikman (1982) are the direct avirulence gene products as well.

The simple organization of the *avr*9 locus which consists of a single open reading frame supports the idea that avr9$^-$ field isolates could have arisen from a single mutation in a previously avr9$^+$ genetic background. Data from Van Kan *et al.* (1991) indeed showed that in two field isolates virulent on *Cf9* plants the *avr*9 gene was deleted.

Karyotype analysis of different races of *C. fulvum* revealed that wild-type races 2.4.9.11 and 2.4.5.9.11, both lacking *avr*9, have a deletion of 500 kb (Talbot *et al.* 1991). Recent data indicate that the deletion is somewhat smaller (R. Oliver, personal communication; G. Van den Ackerveken, unpublished). A similar situation has recently been reported by Panaccione *et al.* (1992) who showed that the TOX2 locus of the maize pathogen *Cochliobolus carbonum,* which is involved in the first step of the synthesis of the cultivar specific HC-toxin, is not found in the genomes of toxin nonproducing (tox^-) strains. In a recent study, Miao *et al.* (1991) demonstrated that the *Pda6* gene from *Nectria haematococca* (encoding a cytochrome P-450 enzyme responsible for the detoxification of the pea phytoalexin pisatin) was on a dispensable B chromosome which did not segregate during meiosis, giving more pda^- progeny than expected.

Since its introduction in tomato breeding lines, the *Cf9* resistance gene has efficiently contributed to the protection of this crop from *C. fulvum.* Although four races virulent on *Cf9* tomato genotypes have been described (Lindhout *et al.* 1989), they have not yet caused any serious problem. This contrasts with several of the other *Cf* genes which have only offered a very temporary protection due to a rapid spread of new virulent fungal races (Hubbeling 1978). This could be explained by a very low mutation rate at the *avr*9 locus, limiting the appearance of infection centers. An alternative explanation could be that the *avr*9 gene is also acting as an indispensible pathogenicity factor in $avr9^+$ races. This does not seem to be the case since the two transformants we analyzed, first, did not display any visible phenotypic alteration compared to their wild-type parents when growing *in vitro* and, second, could infect equally well both $Cf9^+$ and $Cf9^-$ tomato plants. We should, however, stress that the experimental conditions we used (infection of young seedlings, high spore concentrations, high humidity, and constant temperature) are highly favorable to the fungus and may not reflect the environmental conditions usually found in the field. More experiments are needed to assess the competitive ability of the $avr9^-$ strains with respect to spore formation, spore viability, spore dispersal, and all other traits that might affect the spread of a fungal strain under natural conditions. However, caution should be taken in epidemiological studies when this disruption mutant is used, as it may become a successful new virulent race on tomato cultivars containing the hitherto successful resistance gene *Cf9.* The *avr*9 disruption mutants are different from wild-type $avr9^-$ races as they only have one single gene deletion. Wild-type $avr9^-$ races lack besides the *avr*9 gene a significant stretch of flanking sequences. Other genes are possibly present on this deleted DNA that might be involved in fitness or pathogenicity of *C. fulvum*, which might explain why wild-type $avr9^-$ races are not a serious problem in tomato-growing areas.

MATERIALS AND METHODS

Fungal strains, mutagenesis, and growth conditions.

The *C. fulvum* (syn. *Fulvia fulva*) strains used in this study are listed in Table 1. The pyrimidine auxotrophic mutants were selected among 5-fluoroorotic acid (FOA) resistant mutants as originally described for yeast by Boeke *et al.* (1984). Conidial suspensions in water were plated onto minimal medium supplemented with 10 mM of uridine and 2.5 (race 5) or 3 (race 4) mg ml^{-1} FOA; after UV irradiation to give a 60–70% kill, the plates were kept in the dark for 2 wk. FOA-resistant mutants were purified onto fresh FOA plates and subsequently analyzed for uridine auxotrophy.

All strains were routinely maintained on potato-dextrose agar medium (PDA: Merck, Darmstadt, Germany) which was overlaid with cellophane membranes to recover the mycelia for DNA extractions. Czapek Dox (Oxoid, Basingstoke, U.K.) was the minimal medium used to select prototrophic transformants. All cultures were grown at 22° C.

Plant material and inoculations.

The tomato cultivar Moneymaker (MM, susceptible to all races of *C. fulvum*) and the near-isogenic MM line containing the *Cf9* resistance gene were used in this study. Seedling inoculations were carried out according to Talbot *et al.* (1988). Intercellular (apoplastic) fluids were isolated as described by De Wit and Spikman (1982) and injected into the leaves of 6- to 8 wk-old tomato plants as described by the same authors.

Fungal transformation.

Protoplasts of *C. fulvum* were prepared according to Harling *et al.* (1988), with the modifications of Van den Ackerveken *et al.* (1992). Transformations were performed according to Oliver *et al.* (1987) with the following modifications: to 10^7 or more protoplasts in 100 μl of MTC (1 M $MgSO_4$, 10 mM Tris pH 7.5, 10 mM $CaCl_2$) up to 20 μg of plasmid DNA in 10 μl of H_2O were added. After 15 min of incubation at room temperature, 1 ml of PTC (20% polyethylene glycol 6000, 10 mM Tris pH 7.5, 10 mM $CaCl_2$) was added, and the mixture was left at room temperature for an additional 15 min. The solution was then diluted in 5 ml of liquid minimal medium with 0.8 M sucrose and plated in a top agar layer onto plates containing the same selective medium.

DNA manipulations.

Standard DNA techniques used were as described by Sambrook *et al.* (1989). The *E. coli* strain DH5α (F^- φ80d*lac*Z M15 (*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17($r_k^-m_k^+$) *deo*R *thi*-1 *sup*E44 *gyr*A96 *rel*A1) was used to propagate plasmids. *C. fulvum* genomic DNA was extracted from freeze-dried mycelia using the method described by Van Kan *et al.* (1991). DNA from infected leaves was extracted as described by Van der Beek *et al.* (1992). Following restriction enzyme digests, DNA fragments were separated in 0.7% agarose gels and transferred by Southern blotting onto Hybond N^+ membranes using NaOH according to the manufacturer's instructions (Amersham). Membranes were probed with random-primed ^{32}P-labeled DNA fragments, hybridizations and posthybridization washes were carried out at 65° C in aqueous buffers.

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