Cloning and Characterization of cDNA of Avirulence Gene *avr9* of the Fungal Pathogen *Cladosporium fulvum*, Causal Agent of Tomato Leaf Mold

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A race-specific peptide elicitor from *Cladosporium fulvum* induces a hypersensitive response on Cf9 tomato genotypes. We have hypothesized that the avirulence of fungal races on Cf9 genotypes is due to the production of this elicitor by an avirulence gene, *avr9*. To obtain cDNA clones of the *avr9* gene, oligonucleotide probes were designed based on the amino acid sequence determined previously. In northern blot analysis, one oligonucleotide detected an mRNA of 600 nucleotides in tomato-*C. fulvum* interactions involving fungal races producing the elicitor. A primer extension experiment indicated that the probe hybridized to a region near position 270 of the mRNA. The probe was used to screen a cDNA library made from poly(A)$^+$ RNA from an appropriate compatible tomato-*C. fulvum* interaction.

One clone was obtained corresponding to the mRNA detected by the oligonucleotide probe. Sequence analysis revealed that this clone encoded the *avr9* elicitor. By isolating longer clones and by RNA sequencing, the primary structure of the mRNA was determined. The mRNA contains an open reading frame of 63 amino acids, including the sequence of the elicitor at the carboxy-terminus. A time course experiment showed that the *avr9* mRNA accumulates in a compatible tomato-*C. fulvum* interaction in correlation with the increase of fungal biomass. The *avr9* gene is a single-copy gene that is absent in fungal races which are virulent on tomato Cf9 genotypes. Possible functions of the avirulence gene are discussed.

Specialization of plant pathogens has resulted in the evolution of various formae speciales (fungi) and pathovars (bacteria) that are able to colonize only one or a small number of host plants. Within one forma specialis or pathovar, races can be found that are virulent only on certain cultivars of the host, but are avirulent on other cultivars (Brasier 1987). Avirulence is caused by recognition of the pathogen by the host resulting in active host defense (hypersensitive response, HR). The molecular basis of pathogen recognition by the host plant is still largely unknown. Nevertheless, the pathogen must evade or suppress this recognition in order to colonize the plant successfully.

The genetics of many plant-pathogen interactions that are race-cultivar specific can be described by a gene-for-gene model, where an avirulence gene product of the pathogen interacting with the corresponding resistance gene product of the plant triggers an HR, eventually leading to incompatibility (Crute 1985; De Wit 1987). Races of a pathogen lacking a functional avirulence gene do not activate the host defense, leading to successful colonization (compatible interaction). Race-specific avirulence genes have been cloned from various plant pathogenic bacteria

by a shotgun approach, involving screening for acquisition of avirulence by virulent races transformed with genomic clones of avirulent races (Staskawicz *et al.* 1984, 1987; Shintaku *et al.* 1989; Vivian *et al.* 1989; Hitchin *et al.* 1989). Transformants of the virulent races carrying a specific avirulence gene can be detected on cultivars carrying the appropriate resistance gene. For plant pathogenic fungi, such a complementation strategy is difficult to conduct due to larger genome sizes, low efficiency of transformation, and the lack of suitable cloning systems. The only fungus for which an efficient transformation system with autonomously replicating vectors has been established is *Ustilago maydis* (DeCandolle) Corda (Tsukuda *et al.* 1988). For many other plant pathogenic fungi, integrating cloning vectors are available, but autonomously replicating vectors still have to be developed. Therefore, a different approach, not involving random screening of transformants for acquisition of an altered phenotype, is required to study genes involved in pathogenicity and avirulence.

Our work with the tomato pathogen *Cladosporium fulvum* Cooke (syn. *Fulvia fulva* (Cooke) Cif) has resulted in the identification of fungal race-specific elicitors that induce necrosis on tomato cultivars with the corresponding resistance genes (De Wit and Spikman 1982; De Wit *et al.* 1985). One such race-specific elicitor, the putative product of avirulence gene *avr9*, has been purified to homogeneity. The purified protein induced rapid and extensive necrosis when injected into leaves of tomato genotypes carrying the Cf9 resistance gene, but not in genotypes containing other Cf genes (Scholtens-Toma and De Wit 1988). The amino acid sequence of the purified elicitor has been determined (Scholtens-Toma and De Wit

1988). The elicitor was produced in all compatible tomato-*C. fulvum* interactions involving fungal races that are avirulent on Cf9 tomato genotypes, but not in any interaction involving fungal races which are virulent on Cf9 tomato genotypes (Scholtens-Toma et al. 1989). In this study, we report the cloning and characterization of the cDNA from avirulence gene *avr9*, which is to our knowledge the first fungal avirulence gene that has ever been cloned.

MATERIALS AND METHODS

Plants, fungi, and inoculations. Near-isogenic tomato cultivars were grown as described by De Wit and Flach (1979). Different races of *C. fulvum* were subcultured and inoculated onto tomato plants as described by De Wit (1977). The races used in this study and the outcome of their interaction with Cf9 tomato genotypes are shown in Table 1. Table 1 also indicates hypothetical fungal genotypes that would account for these interactions based on the gene-for-gene model.

RNA isolation and northern blotting. RNA was isolated by homogenizing tissue in guanidine-HCl buffer (8 M guanidine-HCl, 20 mM 2-[N-morpholino]ethanesulfonic acid, 20 mM EDTA, 50 mM β-mercaptoethanol, pH 7), extracting with phenol/chloroform (1:1) and chloroform, and precipitating overnight with 2 M LiCl. Poly(A)$^+$ RNA was obtained by affinity chromatography on oligo(dT)-cellulose, electrophoresed on denaturing formaldehyde-agarose gels, and blotted on Hybond N or Hybond N$^+$ membranes (Amersham Nederland BV, Houten, The Netherlands) as described by Maniatis et al. (1982).

Hybridization with oligonucleotide probes and DNA restriction fragments. Oligonucleotides were 5'-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used directly for hybridization. Hybridization with RNA blots was performed in 6× SSC (1× is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate, 100 μg·ml^{-1} calf thymus DNA, 25 μg·ml^{-1} yeast tRNA at 35° C. The blots were washed in 1× SSC, 0.5% SDS at 35° C. Hybridization of filters containing cDNA recombinant bacteriophages was performed in 6× SSC, 0.5% SDS, 0.1% sodium pyrophosphate, 100 μg·ml^{-1} calf thymus DNA at 32° C. The filters were washed under the same conditions.

Table 1. Interaction of *Cladosporium fulvum* races with Cf9 tomato genotypes

Race	Avirulence genotypes	Interaction with Cf9 tomato genotype
2	$A_4A_5A_9A_{11}$	I[a]
4	$A_2A_5A_9A_{11}$	I
5	$A_2A_4A_9A_{11}$	I
2.4	$A_5A_9A_{11}$	I
2.5	$A_4A_9A_{11}$	I
2.4.5	A_9A_{11}	I
2.5.9	A_4A_{11}	C
2.4.11	A_5A_9	I
2.4.5.11	A_9	I
2.4.9.11	A_5	C
2.4.5.9.11	...	C

[a] I = incompatible interaction, fungal race avirulent; C = compatible interaction, fungal race virulent.

Restriction fragments of cDNA clones were isolated from agarose gels and labeled with [α-^{32}P]dATP using a Prime-A-Gene labeling kit (Promega, Madison, WI). Hybridization was conducted at 42° C in 5× SSC, 0.5% SDS, 5× Denhardt's solution (1× is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 100 μg·ml^{-1} calf thymus DNA. Filters were washed at 60° C in 0.2× SSC, 0.2% SDS (high stringency) or at 50° C in 1× SSC, 0.5% SDS (low stringency).

Primer extension and RNA sequencing. Oligonucleotides were 5'-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Five nanograms of labeled primer was hybridized to 5 μg of poly(A)$^+$ RNA in 2× reverse transcriptase buffer (Promega) by heating to 65° C and cooling down to room temperature. The primer was extended with avian myeloblastosis virus reverse transcriptase (Promega) either in the absence or presence of dideoxynucleotides for RNA sequencing. The concentrations of ddNTPs in individual sequencing reactions were 0.2 mM except for ddTTP (0.4 mM).

cDNA synthesis and library construction. cDNA was synthesized on 5 μg of poly(A)$^+$ RNA isolated from the interaction between cultivar Cf5 and pathogen race 5 with the Protoclone cDNA kit (Promega). *Eco*RI sites in the cDNA were modified with *Eco*RI methylase (Promega), and *Eco*RI linkers were added with T4 DNA ligase. After *Eco*RI digestion, excess linkers were removed with a Qiagen tip (Diagen GmbH, Düsseldorf, Federal Republic of Germany), and cDNA was ligated to *Eco*RI-digested λ gt11 DNA (Promega) arms. After ligation, λ phage DNA was packaged with a Packagene kit (Promega) and plated on *Escherichia coli* Y1090 (r$^-$). The primary library (100,000 independent recombinants) was amplified and stored at 4° C.

Cloning procedures and DNA sequencing. All DNA manipulations were conducted essentially as described by Maniatis et al. (1982). DNA sequencing was performed with the chain termination method of Sanger et al. (1977), using [α-^{35}S]dATP label, on double-stranded plasmid DNA with T7 and SP6 promoter primers specific for pGEM plasmids (Promega).

Southern blot analysis of fungal DNA. Total DNA of *C. fulvum* was isolated by grinding freeze-dried mycelium in liquid N$_2$, homogenizing in extraction buffer (0.5 M NaCl, 10 mM Tris-Cl, 10 mM EDTA, 1% SDS, pH 7.5), extracting with phenol/chloroform (1:1) and chloroform, and precipitating the aqueous phase with isopropanol. DNA was treated with RNase and digested with *Eco*RI, *Hind*III, *Pst*I, or *Xho*I. From each race, approximately 5 μg of digested DNA was electrophoresed on a 0.7% agarose gel. DNA was depurinated, denatured, and blotted in 10× SSC onto Hybond N$^+$ with a vacuum blotter (Millipore BV, Etten-Leur, The Netherlands).

RESULTS

Design of oligonucleotide probes and screening on northern blots. To detect the mRNA encoding the race-specific elicitor, four oligonucleotide probes were synthesized, derived from the amino acid sequence shown in Figure 1. The oligonucleotides contained either mixtures

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  1    2    3    4    5    6    7    8    9   10   11   12   13   14   15   16   17   18   19   20   21   22   23   24   25   26   27
 Tyr  Cys  Asn  Ser  Ser  Cys  Thr  Arg  Ala  Phe  Asp  Cys  Leu  Gly  Gly  Cys  Gly  Arg  Cys  Asp  Phe  His  Lys  Leu  Gln  Cys  Val

 UAU  UGU  AAU  UCU  UCU  UGU  ACU  CGU  GCU  UUU  GAU  UGU  CUU  GGU  GGU  UGU  GGU  CGU  UGU  GAU  UUU  CAU  AAA  CUU  CAA  UGU  GUU
  C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    C    G    C    G    C    C
                 A    A         A    A    A              A    A    A         A    A                        A              A
                 G    G         G    G    G              G    G    G         G    G                        G              G
               AGU  AGU            AGA            UUA                  AGA                           UUA
                 C    C              G              G                    G                             G
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PROBE A: 5' CC ACA TTG ICC IAG ACA ATC AAA 3' (a.a. 10-17)
              G   C   A G   G   G
PROBE B: 5' AG TTT ATG AAA ATC ACA 3' (a.a. 19-24)
              A C   G   G   G
PROBE C: 5' AC ICA ITG IAG TTT ATG AAA ATC ACA 3' (a.a. 19-27)
                  A   C   G   G   G   G
PROBE D: 5' AC ICA ITG IAI ITT ITG IAA ITC ICA 3' (a.a. 19-27)

Fig. 1. Amino acid sequence of the race-specific *avr9* elicitor with the corresponding coding sequences. The four oligonucleotide probes (A–D) used in this study are shown below. Degeneration of the probes at ambiguous positions was obtained by incorporating mixtures of dNTPs as indicated. In probes A, C, and D, inosine (I) was incorporated in some positions.

of nucleotides (as in probe B) or inosines (as in probe D) at ambiguous positions, or a combination of both (as in probes A and C). All four oligonucleotides were 5'-end-labeled and hybridized to identical northern blots containing equal amounts of poly(A)$^+$ RNA from uninoculated tomato plants, from *C. fulvum* grown *in vitro*, and from three different compatible tomato-*C. fulvum* interactions. Figure 2 shows that probe B hybridized specifically with an mRNA of approximately 600 nucleotides present in two compatible interactions: cultivar Cf4 with pathogen race 4 (lane 3) and cultivar Cf5 with pathogen race 5 (lane 4). This mRNA was not detected in uninoculated cultivar Cf5 (lane 1) or in *C. fulvum* grown *in vitro* (lane 2). Also, no hybridization was observed in the interaction between Cf5 and race 2.4.5.9.11 (lane 5), as would be expected for an interaction involving a race that is virulent on Cf9 tomato genotypes. Thus, the preliminary conclusion was that probe B detected the mRNA for the necrosis-inducing peptide. Probes A, C, and D did not detect any specific mRNAs on blots identical to the one shown in Figure 2 (results not shown).

Oligonucleotide probe B was used in a primer extension experiment. The oligonucleotide was 5'-end-labeled and hybridized to equal amounts of poly(A)$^+$ RNA isolated from compatible interactions of cultivar Cf5 with either race 5 or race 2.4.5.9.11 (represented in Fig. 2, lanes 4 and 5, respectively). The primer was extended with reverse transcriptase, and the extension products were analyzed on a denaturing polyacrylamide gel. Figure 3 shows that a specific extension product was synthesized on poly(A)$^+$ RNA from the interaction between cultivar Cf5 and race 5 (lane 1), but not on poly(A)$^+$ RNA from the interaction between cultivar Cf5 and race 2.4.5.9.11 (lane 2). The length of the extension product is approximately 270 nucleotides, indicating that the *avr9* mRNA contains approximately 200 nucleotides upstream of the sequence encoding the necrosis-inducing peptide.

Preparation and initial screening of the cDNA library. Poly(A)$^+$ RNA from the interaction between cultivar Cf5 and race 5 (represented in Fig. 2, lane 4) was used to prepare a cDNA library in λ gt11. A library was obtained containing 100,000 independent recombinants. Screening of filters containing 5,000 phages with 5'-end-labeled oligonucleotide
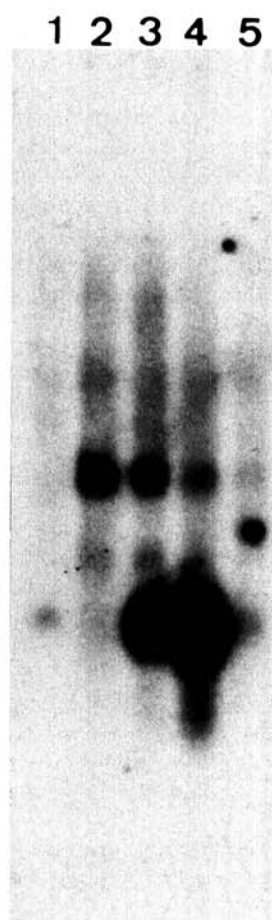
1 2 3 4 5

Fig. 2. Northern blot hybridization with oligonucleotide probe B. Probe B was 5'-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and hybridized to a northern blot containing equal amounts of poly(A)$^+$ RNA isolated from uninoculated tomato cultivar Cf5 (lane 1), *Cladosporium fulvum* grown *in vitro* (lane 2), or from three compatible tomato-*C. fulvum* interactions: Cf4 with race 4 (lane 3), Cf5 with race 5 (lane 4), and Cf5 with race 2.4.5.9.11 (lane 5).

probe B resulted in the isolation of two possible candidates, one hybridizing weakly (phage A9-1), the other hybridizing significantly better (phage A9-2). Both phages were purified and their DNA was isolated. The phage DNAs were labeled by random-primed labeling and hybridized with blots

identical to the blot shown in Figure 2. Phage A9-1 hybridized with an mRNA of low abundance of approximately 1,500 nucleotides present in all three tomato-*C. fulvum* interactions (result not shown). This phage did not contain a cDNA corresponding to the mRNA detected by northern blotting (Fig. 2), and was not analyzed further.

Labeled DNA from phage A9-2 hybridized with an mRNA of approximately 600 nucleotides present only in the compatible interactions between Cf4 and race 4 and between Cf5 and race 5, that is a pattern identical to the hybridization observed with oligonucleotide probe B (result not shown). Thus, phage A9-2 contained a copy of the mRNA from the *avr9* gene, encoding the necrosis-inducing peptide. Restriction analysis of the DNA from phage A9-2 indicated that the cDNA was flanked by only one *Eco*RI site, the other *Eco*RI site had been lost during cDNA

Fig. 3. Primer extension with oligonucleotide probe B. Probe B was 5'-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, hybridized to 5 μg of poly(A)$^+$ RNA, and extended with avian myeloblastosis virus reverse transcriptase. The extension products were analyzed on a denaturing 6% polyacrylamide gel. Lane 1 contains the extension products synthesized on poly(A)$^+$ RNA from the interaction between cultivar Cf5 and pathogen race 5, and lane 2 contains the extension products synthesized on poly(A)$^+$ RNA from the interaction between Cf5 and race 2.4.5.9.11. Lane 3 contains marker fragments, the lengths of which are given in nucleotides in the margin.

cloning. The insert was estimated to be 400 base pairs.

Sequence analysis of the *avr9* cDNA. The cDNA insert present in phage A9-2 was subcloned and the sequence was determined. The insert is 405 base pairs long and corresponds to the 3' end of the mRNA, including a poly(A) tail of 20 nucleotides. The insert encodes the entire sequence of the necrosis-inducing peptide contained within a longer open reading frame of 63 amino acids. From the position of the oligonucleotide probe B in the DNA sequence and the size of the primer extension product, it was estimated that the insert of clone A9-2 lacks about 110 nucleotides of the 5' end of the mRNA. To obtain full-length cDNA clones, the cDNA library was screened again with a labeled RNA probe containing 70 nucleotides of the 5' end of the insert of clone A9-2. Three different phages (A9-3, A9-5, and A9-8) were obtained, and their inserts were subcloned and sequenced. Clone A9-3 did not contain an *Eco*RI site at the 3' end of the cDNA insert and was, therefore, only sequenced from the 5' end. The sequence of all three clones was completely identical to the sequence of clone A9-2 in the overlapping regions. All three clones containing poly(A) tails have different sites of polyadenylation. From the DNA sequence analysis and the primer extension experiment shown in Figure 3, it was deduced that clone A9-3, which contained the most prolonged 5' sequence of the mRNA, still lacked approximately 35 nucleotides. Therefore, a new primer was designed, hybridizing at position 75–100. This primer was used in a primer extension experiment on poly(A)$^+$ RNA in the presence of dideoxynucleotides. The RNA sequencing allowed the addition of another 24 nucleotides upstream of the insert of A9-3 (results not shown). The first seven nucleotides of the *avr9* mRNA could not be read. Three minor extension products were observed that were five to 20 nucleotides longer than the major extension product. The different end products of the primer extension were not a result of degradation of mRNA, since an extension experiment with a primer for a different fungal mRNA yielded one discrete extension product of the correct length (results not shown). The structure of the *avr9* cDNA clones and the sequence of the *avr9* mRNA are shown in Figure 4.

Timing of expression of the *avr9* mRNA. To determine the timing of expression of the *avr9* mRNA, a northern blot was made containing equal amounts of poly(A)$^+$ RNAs from tomato leaves harvested at different times after inoculation with *C. fulvum*. One time course was made of the compatible interaction between Cf5 and race 5, and another one of the incompatible interaction between Cf4 and race 5. Poly(A)$^+$ RNAs from uninoculated tomato (cultivar Cf5) and from *C. fulvum* grown *in vitro* (race 5) were included as controls. The blot was hybridized with the labeled insert of clone A9-2. Figure 5 shows that the *avr9* mRNA could be detected in the compatible interaction at day 6 postinoculation, strongly increasing from day 8 postinoculation and onward. No hybridization could be observed in the incompatible interaction. A small amount of mRNA could be detected in the fungus grown *in vitro* (Fig. 5, lane F). However, when comparing mRNA concentrations of the fungus grown *in vitro* with the fungus grown *in planta*, it should be considered that the RNA samples from the compatible interaction contain only small
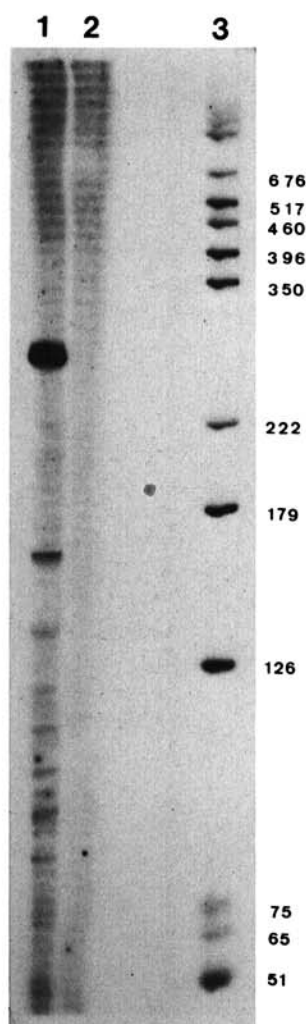
proportions of fungal mRNAs, especially in early stages of infection. Relative to this, only very minute amounts of fungal RNA are obtained from incompatible interactions, since fungal growth is inhibited completely shortly after penetration of the plant.

Southern blot analysis of the *avr9* gene. Southern blot analysis of DNA isolated from seven races of *C. fulvum* (Fig. 6) indicated that the cDNA clone hybridized to single bands in various restriction enzyme digests. The *avr9* gene is present in a single copy in races 2, 4, 5, 2.4, and 2.4.5 (Fig. 6, lanes 1, 2, 3, 5, and 6), respectively. The gene could not be detected in races 2.5.9 and 2.4.9.11 that are virulent on genotype Cf9 (Fig. 6, lanes 4 and 7). Analysis of four additional races confirmed the presence of single hybridizing fragments in races 2.5, 2.4.5.11, and 2.4.11 and the absence of hybridization in race 2.4.5.9.11 (results not shown). The use of a longer cDNA probe (clone A9-5) or lower stringency conditions during hybridization gave hybridization patterns identical to the blot shown in Figure 6 (results not shown). Apparently, the coding sequence of the *avr9* gene is present in races that are

avirulent on Cf9 tomato genotypes and absent in races that are virulent on Cf9 tomato genotypes.

DISCUSSION

Using an oligonucleotide probe, we have detected the mRNA and cloned the gene for the necrosis-inducing peptide, which we hypothesize is encoded by the avirulence gene *avr9* of *C. fulvum*. We believe that this gene is the avirulence gene, since its gene product specifically induces HR on Cf9 tomato genotypes. Nevertheless, it remains to be proven that transformation of *C. fulvum* races, lacking *avr9*, with the *avr9* gene confers avirulence on Cf9 tomato genotypes in a gene-for-gene relation. The isolation and characterization of cDNA clones revealed that the necrosis-inducing peptide is produced as a precursor protein of 63 amino acids. Surprisingly, the DNA sequence indicated an additional histidine codon at the carboxy-terminus of the sequence of the mature elicitor. The elicitor was reported previously to be 27 amino acids long (Scholtens-Toma and De Wit 1988), but reexamination of the protein sequence
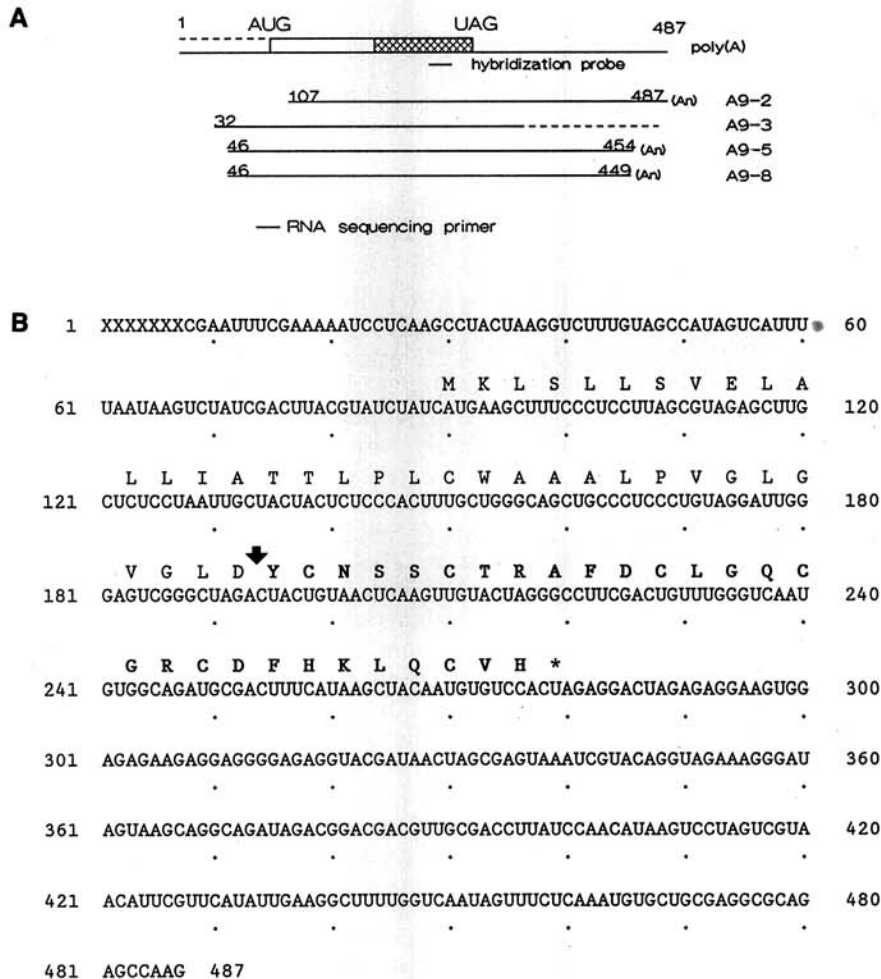

Fig. 4. A, Structure of *avr9* cDNA clones. The crosshatched block indicates the amino acid sequence of the mature *avr9* elicitor, and the open block indicates the putative signal peptide. Beginning and polyadenylation sites of three cDNA clones (A9-2, A9-5, and A9-8) are indicated by numbers corresponding to the position in the mRNA. Clone A9-3 was not sequenced from the 3′ end. **B,** Nucleotide sequence of the *avr9* mRNA and its encoded protein. In the amino acid sequence, the mature necrosis-inducing peptide is indicated in bold characters; the cleavage site in the precursor is shown by an arrow.

data confirmed the presence of an extra histidine residue at position 28. The signal of histidine had been overlooked during initial protein sequence analysis. The molecular mass of the mature *avr9* elicitor is 3,192 Da. The presence of the extra histidine probably explains why a synthetic peptide of 27 amino acids (lacking the carboxy-terminal histidine) was not biologically active in bioassays on Cf9 tomato genotypes (P. J. G. M. de Wit and F. Th. Brederode, unpublished results). The peptide was poorly soluble in water. Several attempts have been made to activate the synthetic peptide of 27 amino acids by reduction and slow oxidation, but we never obtained a preparation that was biologically active. Usually, the removal of ureum and/or acetic acid from the solvent by dialysis made most of the peptide precipitate. The supernatant contained no necrosis-inducing activity (P. J. G. M. de Wit and F. Th. Brederode, unpublished results). The biological activity of the chemically synthesized peptide of 28 amino acids will be tested in future experiments.

The amino-terminal amino acids that are absent in the mature elicitor have some characteristics of a signal peptide (length and hydrophobic characteristics). The cleavage site (between an aspartate residue and a tyrosine residue), however, does not correspond to the (−3,−1) rules for cleavage of signal peptides (Von Heijne 1986). Probably, the putative signal peptide is removed during excretion from the fungal cell into the apoplast, but it remains to be determined whether plant factors are needed for additional maturation to a biologically active elicitor.

The A9 mRNA could be detected at day 6 post-inoculation, that is approximately 3 days after penetration of the stomata by fungal hyphae. The mRNA increases very rapidly between day 6 and day 8 postinoculation and onward. This increase might reflect the rapid increase in fungal biomass during this period, as deduced from two parameters for fungal biomass *in planta*, mannitol concentration and mannitol dehydrogenase activity in apoplastic fluids of infected leaves. These two parameters show a rapid increase between day 6 and day 8

postinoculation (Joosten *et al.* 1990). Before day 6 postinoculation, no significant fungal biomass could be measured in compatible interactions, and consequently, no *avr9* mRNA could be detected. Also, in the incompatible interaction, no fungal biomass and no *avr9* mRNA could be detected (Fig. 5). Nevertheless, it is very likely that the *avr9* gene is expressed at very early stages of the infection cycle, since its gene product is believed to trigger the HR on Cf9 tomato genotypes at about day 4 postinoculation (within 1 day after penetration of the stomata). Final evidence about the expression of *avr9* mRNA in early stages of infection will be obtained from *in situ* hybridization experiments.

It is difficult to speculate on a biological function of the necrosis-inducing peptide for the fungus. From the point of view of the fungus, the possession of an *avr* gene would be disadvantageous, unless the gene has some beneficial function. Therefore, we propose that this peptide has another, however dispensable, function in the infection cycle of *C. fulvum*. The function of the peptide is either
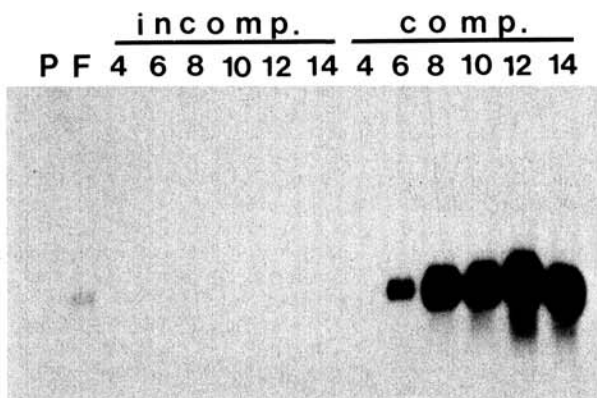
Fig. 6. Southern blot analysis of total DNA from different races of *Cladosporium fulvum*. Races used were race 2 (lane 1), race 4 (lane 2), race 5 (lane 3), race 2.5.9 (lane 4), race 2.4 (lane 5), race 2.4.5 (lane 6), and race 2.4.9.11 (lane 7). Five micrograms of fungal DNA was digested with *Eco*RI, *Hind*III, *Pst*I, or *Xho*I as indicated, separated on a 0.7% agarose gel, and blotted onto Hybond N⁺ membranes. The blot was hybridized with a random-primed, labeled fragment of 420 nucleotides containing the entire cDNA insert present in phage A9-2.

Fig. 5. Time course of accumulation of the *avr9* mRNA. The insert of clone A9-2 was labeled and hybridized to a northern blot containing equal amounts of poly(A)⁺ RNA isolated from uninoculated cultivar Cf5 (lane P), *Cladosporium fulvum* grown *in vitro* (lane F), an incompatible interaction between cultivar Cf4 and pathogen race 5 (lanes incomp.), or from a compatible interaction between Cf5 and race 5 (lanes comp.) at different times (4, 6, 8, 10, 12, and 14 days, respectively) after inoculation as indicated.
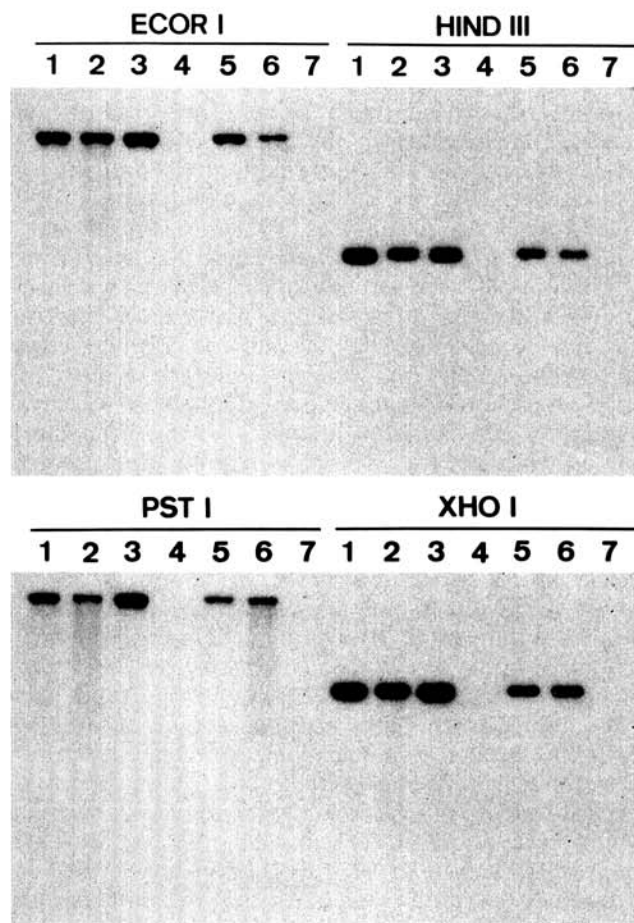
nonessential or can be compensated by other proteins. Whatever its function may be, the mode of action of the *C. fulvum avr9* gene product differs in several aspects from bacterial avirulence gene products. First, the mature *avr9*-encoded protein itself directly induces an HR on Cf9 tomato genotypes (Scholtens-Toma and De Wit 1988). Purified proteins encoded by bacterial avirulence genes do not induce the HR directly (Tamaki *et al.* 1988; Ronald and Staskawicz 1988), but rather seem to act via the production of low molecular weight elicitor-active molecules (Keen *et al.* 1990). Second, the *avr9* gene product is a protein that is excreted by the pathogen into the apoplast of infected leaves. In the case of bacterial avirulence gene products, all evidence points to an intracellular location of these proteins (Napoli and Staskawicz 1987), thus reinforcing the hypothesis that they produce elicitor-active compounds (Keen *et al.* 1990).

Fungal races that do not produce the *avr9* elicitor, and are consequently virulent on Cf9 tomato genotypes, entirely lack at least the coding region of the *avr9* gene. Hybridization with flanking sequences of the gene (isolated from a genomic library) should reveal whether these races lack more than just the coding sequence. Accordingly, these races do not contain a nonfunctional allele that could overcome the avirulent phenotype. Therefore, the assignment of virulence gene 9 could be regarded as misleading. The absence of the *avr9* gene in *C. fulvum*, resulting in virulence on a formerly resistant host, is analogous to the absence of three avirulence genes (*avrA*, *avrB*, and *avrC*) in virulent races of *Pseudomonas syringae* pv. *glycinea* (Coerper) Young *et al.* (Staskawicz *et al.* 1984, 1987). In contrast, nonfunctional alleles of avirulence genes were demonstrated in the plant pathogenic bacteria *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye (Kearney *et al.* 1988) and *P. s.* pv. *glycinea* (Kobayashi *et al.* 1989, 1990b). In spontaneous mutants of *X. c.* pv. *vesicatoria*, which had lost a functional *avrBs1* gene, most if not all mutants appeared to contain a transposable element, either in the coding region or in regulatory sequences (Kearney *et al.* 1988). In the case of the *avrD* gene isolated from *P. s.* pv. *tomato* (Okabe) Young *et al.*, other pathovars of *P. syringae* (e.g. *P. s.* pv. *glycinea*) appeared to have a homologous allele (Kobayashi *et al.* 1989, 1990b) that seems to be a functional gene (with 86% amino acid homology to *avrD*) lacking the necrosis-inducing phenotype (Kobayashi *et al.* 1990b).

It is tempting to speculate about the origin of the *avr9* gene. It has been shown that some avirulence genes of plant pathogenic bacteria reside on endogenous plasmids (Kobayashi *et al.* 1990a; Swanson *et al.* 1988). Several plasmids have been detected in plant pathogenic fungi (Leong and Holden 1989), and recent data have indicated the presence of pathogenicity genes on so-called B chromosomes (Van Etten *et al.* 1989). The possible presence of the *avr9* gene on an episomal factor or a B chromosome should be considered. Alternatively, it is possible that the *avr9* gene is located in an unstable region of the genome which can be deleted without dramatically affecting the viability of the fungus. Experiments to distinguish between a genomic or episomic localization of the *avr9* gene are currently being conducted. It remains to be determined whether virulence of other, if not all, fungal pathogen races on various other Cf tomato genotypes is caused by deletion of the corresponding avirulence genes. We are currently purifying race-specific elicitors other than A9 in order to clone their encoding genes and determine whether loss of avirulence genes is a general feature in virulent phenotypes. It will be interesting to determine whether avirulence gene products might have a beneficial effect in some stages of the fungal life cycle. Competition experiments between a race lacking *avr9* and a transformant of this race containing the gene could give a clue about the relevance of the *avr9* gene for *C. fulvum*.

It will be of great interest to isolate and characterize the entire *avr9* gene with its regulatory elements, so we can study the regulation of this gene *in vivo* in response to plant signals. Preliminary results indicate that under certain defined growth conditions expression of the *avr9* gene can be induced *in vitro* (R. M. Dunn, W. Tÿs, and P. J. G. M. de Wit, unpublished results). The structure and regulation of expression of the *avr9* gene will be an object of further studies.

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