

Characterization of Two Putative Pathogenicity Genes of the Fungal Tomato Pathogen *Cladosporium fulvum*

Guido F. J. M. Van den Ackerveken, Jan A. L. Van Kan, Matthieu H. A. J. Joosten, José M. Muisers, Henk M. Verbakel, and Pierre J. G. M De Wit

Department of Phytopathology, Agricultural University, Wageningen, The Netherlands.
Received 29 July 1992. Accepted 14 December 1992.

The fungus *Cladosporium fulvum* is a biotrophic pathogen of tomato. On susceptible tomato plants, the fungus grows abundantly in the extracellular spaces between the mesophyll cells. The mechanism by which *C. fulvum* is able to establish and maintain basic compatibility on its one and only host species, the tomato, is unknown. The isolation and characterization of pathogenicity factors and the corresponding genes will provide insight into the mechanism by which *C. fulvum* colonizes its host. Two putative pathogenicity genes of *C. fulvum* encoding proteins, which occur abundantly in the extracellular space of infected tomato leaves, were isolated and characterized (*ecp1* and *ecp2*). The DNA sequences of these *ecp* genes (encoding extracellular protein) do not share homology to any sequence present in the DNA databases. The *ecp* genes are highly expressed *in planta* but not *in vitro*, suggesting that they play a significant role in pathogenesis.

Additional keywords: basic compatibility extracellular protein, fungal pathogenicity.

The interaction between the biotrophic fungal pathogen *Cladosporium fulvum* Cooke and tomato (*Lycopersicon esculentum* Mill.) is confined to the leaf surface and the extracellular space between the mesophyll cells. No specialized structures, involved in penetration and nutrient uptake, could be detected microscopically (De Wit 1977; Lazarovits and Higgins 1976a, 1976b). The pathogen is able to grow in the extracellular space without destroying host cells. Studies on the carbohydrate composition of the apoplastic fluid of *C. fulvum*-infected leaves indicated that the fungus is probably dependent on extracellular sucrose as its main carbon source (Joosten *et al.* 1990).

Incompatibility between *C. fulvum* and tomato is caused by the recognition of fungal avirulence gene products, the so-called race-specific elicitors, by the resistant host, leading to the activation of the hypersensitive response (HR), which restricts the pathogen to the site of infection (Van den Ackerveken *et al.* 1992). In compatible interactions

no HR is induced and other defense responses, such as the accumulation of PR proteins, are activated much later than in incompatible interactions (Joosten and De Wit 1989). The accumulation of pathogenesis related (PR) proteins and other compounds in compatible interactions is most probably induced by general stress and possibly by cell wall fragments released from the fungus during pathogenesis.

The way *C. fulvum* colonizes tomato, without the formation of specialized structures and the production of damaging hydrolytic enzymes, raises questions about the mechanism by which the fungus establishes and maintains basic compatibility. The isolation and characterization of putative pathogenicity factors and their corresponding genes should provide an insight into the strategy adopted by *C. fulvum* to successfully infect its one and only host species, the tomato.

In compatible *C. fulvum*-tomato interactions several low molecular weight proteins (<20 kDa) accumulate in the extracellular space during the infection process (De Wit *et al.* 1989). These proteins are thought to be of fungal origin, as they are not detected in healthy plants nor in incompatible *C. fulvum*-tomato interactions. These putative pathogenicity factors might play an important role in establishing basic compatibility. Two such extracellular proteins, ECP1 (synonymous with P1; Joosten and De Wit 1988) and ECP2 (J. P. Wubben, unpublished) have been purified and polyclonal antisera have been raised against these proteins. Western blot analyses indicated that proteins ECP1 and ECP2 are neither present in *C. fulvum* grown *in vitro*, nor in healthy tomato plants. In this paper we describe the isolation and characterization of the genes *ecp1* and *ecp2*. They are shown to be of fungal origin and are highly expressed in *C. fulvum*-infected tomato leaves, whereas their expression *in vitro* is low or not detectable.

RESULTS

Isolation of the *ecp1* gene.

The ECP1 protein was purified as previously described (Joosten and De Wit 1988), and the sequence of the 36 N-terminal amino acids was determined. A degenerate oligonucleotide probe was designed, complementary to the derived mRNA sequence encoding amino acids 27–32 (Fig. 1). By using this probe on Northern blots, we detected a clear hybridization signal specifically in RNA isolated from compatible *C. fulvum*-tomato interactions (results

Address correspondence to: Pierre J. G. M. de Wit, Department of Phytopathology, P. O. Box 8025, 6700 EE Wageningen, The Netherlands.

MPMI Vol. 6, No. 2, pp. 210-215

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1993.

CTGCAGTGTGCCATGCGCTTATTACAGACGACTTCTCGGGGATACTCGCAGGAAGTGGAC -605
 GTTCGTGCTTTGATGAGTTGAAGCACTCCCCGAGAGTCGACAGCCGATTGTGACCCGTCAAT -545
 TGGCCTCCACGTTCCGGTTCGCGCATTTCGAGCCGGGATGGGTTGGAAAGTCCGCTG -485
 TTAAGAAAGCGGTAGTTGCCATTCTCGGGCGTCACTTCGGGGCCGCGCGGATTTAGCT -425
 CATCGAGCCAGGTTGCCACATGCCCTCCCACTGCAACGATCATGTTGGTACGAAG -365
 CGCAGAACACTGCCTTTACGAGACTCGTAACCTGTAACATCTACCAAGTCACTCGA -305
 ATCTCGCCATCTCGCAAGCGCAGGGTATGCTCTCACGACCAGGATAAGTTCGCACT -245
 CCGGACGGCGTATTTACCAAGCAAGCTTACGAGATGGCCGCGATTTCGAGATGACCCTGGTA -185
 GATGTTATA**CAAT**GAGTTGCGGAACCTTCGCGGACCTTCGTAATATTGTGACGCGTACTGT -125
 ACAATGCTTGATGTAAGCTTGGCACAACATCCAAGAAGCCTGATCGGTGCGGCCAGTGTCT -65
 TGTAGACGGCACCTAGGTGTGCAACCT**TATAAAG**CAGCCCGTCTGCGCTGCGCAATTATG -5
 CTTCATCACATCCTTAGTTACCGAGACATCAACACTCCCACTCCCTCCAAG**ATGC**ACTT 56
 M H F
 CGTCACATCTCTCATCGCCGGCTCGCCATGCTGCCACGGGAGCGCCACTGTCCAAG 116
 V T S L I A G V A M L A T A S A T V Q G
 CGGGCCCCCGTGGACGATCTCAAGTTCGCAAAAAGTTC AAC CAGA ACTGCCAGCAAT 176
 G A P V D D L K F A K K F N Q H C Q Q I
 CTCGTGGCGTCCAACGGGTAAAGCCCTCGACCCCTCATCCAACCCACTCGGCCACAATAG 236
 S G G P N G -----
 ATCGGAAGACTCCACGAACTAACACGAATGATACCATCTTAGAGCGATCTGCCAGAC 296
 ----- A I C P D
 GGGACTTGGTATGACCACCTCGTACCCCTCAGCGATCGATGCTCTCGAACTAATGG 356
 G D L
 GGAATGCCCGGGACAGTATTGGTGC AAAGAGCGGTAGGCCATATTTTGGCAAGACATGC 416
 -----Y W C K D G R A I F C Q T C
 CAAACTGGATGTAACCGCGATGAAAATGGCAAGTCCGGTACTGCAACGAAGGCCCTACA 476
 Q T G C T A D E N G K V G Y C H E G P T
 AACCCCAAGTGCCTT**TAAT**GGAGGAAGCATCGCGGAGGCTGAATGCCACCGTTACCAATT 536
 N P K C L
 GAGACGATGTGGAGAATGTGCCCTTCTACGTCGAGGGGAGAGCGACCGTACGCTTACG 596
 TAGACATCCAAGCAACGAGGAGACGTTAAGCCCTGTAATTGCTCAAATCGTCTATCCCTC 656
 TAGATGCCACGCTTCCCTTCGCTAAAGCAAAAGCTGCGCATCTGTGAGCGCAACGACTC 716
 CAGCTCGGGCTGCAGTGGACCGCTCGTCGATGATTTGGGACGACAGCTTCCGCCCCAT 776
 CCTCTACACAAAGCAGACCGGTTTATACAGCCACACTAGCCAGTACGGCCCCATACA 836
 GCGAGATCGTCGCACTAAACATTAGCGAAGAGCATGCCACGGCTACATGTCTTTCGC 896
 CCTGTAGTAGACAAACCGCAGACTTCTACATTTATTGAGCGCAACGACCGACGAGCGCTA 956
 GCCACCCGTAGCGACGTATAAGCTT 982

Fig. 3. Sequence of the *Cladosporium fulvum* gene *ecp1* (EMBL accession number Z14023). The gene encodes a precursor protein of 96 amino acids and is interrupted by two short introns (---). The predicted signal sequence cleavage site is indicated by an arrow (↓). The mature ECP1 protein of 65 amino acids is located at the carboxyl-terminus of the precursor protein (bold). A putative TATA box (TATAAA) is located 37 bp upstream of the main transcription start (▼). The 5' upstream region contains a putative CAAT box (overlined) located at position -175. In the mRNA, the 3' untranslated region of 170 bp is followed by a poly(A) tail starting at the position indicated (▽).

GATCGGAGGTGAACAGAGACTGTAGGTGTTTATGAGACGGGGCAATCTAGTGCAGGC -630
 TGTGGTTAGCCGAAAATCGTGTCAATCGTCTGTCAAATAGCATCGTCAAGGGTTCAG -570
 CCAAGGTTTCTTGTCTCAGATGGAAACTAGCGGGACTATTGGCGGTGAGTCTTCAGA -510
 GGGATGCAAAGTGGATGCTCGTACTCCAGGTCGTGACCGGCACGTGGCCTACTCAGAA -450
 CAACGAGTGTCCACCCTTTAGCAACATACAGCGTCTGTGACGACGGAGAACATCTTTAC -390
 TTAGCGTTCGAGTGTTCGCGCGCGGATGCTATGATCTGCTTCCGCTGGGATAAGA -330
 CGATCTGCACTAACGGGAAATGTAACCTCGCTGCTGACAGACGCTGTACGATTCCAAGAC -270
 GAAGGAGTTTACCGTCTAGGTGTCAAATCTAGATCCCAAGAAAATCCAAATCTGGACCT -210
 TGGCTACATGAGTTACGAAGCAGGTTGCAACAGTGCACCTGCCAAGTCAACGCTATGCG -150
 CTGGTAGGGCGTTTTCGCGATGCCAGTCCAAGATCGTGTCTCAAGATGCAGCCTTA -90
 CAGTTCGAT**CAAA**TCGCCACCAGAAATGACCTCGAGTGTGGGAACGGTATT**TATAGT**C -30
 CCCTAAGTATGGCCCTCAACGCCACCA**AAAG**TTTACCTTACCAGAACTTCCGAACACAGTA 31
 CACTCTCCACACACACCCTCTTCTCTGTGCCAACTCACAGACCATACAACCAGC 91
 TTCAGT**AGT**CTCTTCAACGCCCGCGCCGCGCGTGTTCGCCCTCTGCTAGTCTATGGCC 151
 M L F N A A A A A V F A P L L V M G
 AACGTTCTGCTCGGAACCTGGCAACTCGCCCGCTCAACCGCTGCCATGCCCTCAACT 211
 N V L P R N A G H S P G S H R C D A S T
 TTTAACAAAGCGCAAGACTTTGACATTCACAGGCACAGTCAATGACTGCCGAGATG 271
 F N N G Q D F D I P Q A P V N D C R Q M
 GTTGAACATCAATAGAGATTCTCAATCTCAGTGTCCACAGTGGCGAGACCATTTC 331
 V E N I N R D S Q F S V S H S W A R P F
 GCGGATATGGCGATTGTACGTCACAGAGCTCCACGCAATCTTGAATATACTGACA 391
 G G Y G D C -----
 TACCATTTCAGTGCATTCACCTCCGCTCATTGCTGGTGGCGGAATGGCTTGGTTG 451
 ----- A F H V R V I A G W R H G L V G
 GCGGTGCGGATCGCCTTGACCTTCTCACTGATCTGTGCAAAACTTCGGTGAAGCCACA 511
 G A D A V D L L T D S V K N F G E A N K
 AGGTTTCAGCAAGGGCACCTACAACAAATTGTCTCCGCGGAGGGCGAAGTTACGTGCG 571
 V S S K G T Y N Q I V S A E G E V T C D
 ATTCTGGATCGTGGTGGTCAAGTTCAGTGTGATGTTGGCCAGCTCATCGTACA 631
 S V D R G G Q V R V Q W I V A S S S Y N
 ACCCGTCCACGATGACTAGAGGCCCGCTCAGACGGGTTGTGAGAACCAGGTTGTAGCA 691
 P S N D D
 GAGCTGAACATGGCATTCTGGTGTGCAAGCTTGGCAATCAAAATACAGAGCAACG 751
 GAATGGATCTTGAAGCTAGTAGTCAACCATACGACTGTATCTTTCAACACACACAGCTC 811
 TGATACAGACATCAGTTCGGCATCCACTAAAGCCCTTCGATAACCGTCCATGCCTC 871
 CAGCCTGATTCGAGAATGTGCAACCAACCATCATCTCTGGTCTGCTTCGGCAGCAG 931
 TCATGATC 939

Fig. 4. Sequence of the *Cladosporium fulvum* gene *ecp2* (EMBL accession number Z14024). The gene encodes a precursor protein of 165 amino acids and is interrupted by one short intron (---). The predicted signal sequence cleavage site is indicated by an arrow (↓). The mature ECP2 protein of 142 amino acids is located at the carboxyl-terminus of the precursor protein (bold). A putative TATA box (TATAGT) is located 36 bp upstream of the main transcription start (▼). The 5' upstream region contains a putative CAAT box (overlined) located at position -80. In the mRNA, the 3' untranslated region of 125 bp is followed by a poly(A) tail starting at the position indicated (▽).

at different times after inoculation (Fig. 5). No hybridization was detected in healthy plants nor in incompatible interactions. The *ecp2* transcript is present at a low level in *in vitro*-grown *C. fulvum*, whereas the *ecp1* transcript could not be detected under these conditions. The time course of accumulation of the *ecp1* and *ecp2* transcripts is similar and follows the increase of fungal biomass in the infected leaves. The expression of the *ecp* genes in the compatible interaction, as compared to the fungus grown *in vitro*, is highly induced, taking into consideration the minor proportion of fungal RNA in the infected leaf samples. The *C. fulvum* actin gene (*act*) was used as a constitutive control to determine the presence of fungal mRNA in the infected tomato leaves (G. F. J. M. Van den Ackerveken, unpublished). The *act* transcript is present in *C. fulvum* grown *in vitro* but absent in the incompatible interaction because of the low fungal biomass in the leaf. The level of *act* transcript increases during the compatible interaction following the increase in fungal biomass but stays significantly lower than the level of *in vitro* grown *C. fulvum*.

Further characterization of proteins ECP1 and ECP2.

The Tricine-SDS-PAGE system (Schägger and von Jagow 1987) was applied to determine the molecular weight of ECP1 protein. The conventional SDS-PAGE system was not suited for the separation of proteins smaller than 14 kDa. Acrylamide gels of 16.5%T and 6%C enabled us to clearly separate proteins in the 5–20 kDa range. The molecular weight of the ECP1 protein was estimated to be 9 kDa, which is in agreement with the molecular weight deduced from the DNA sequence.

To confirm that the cloned *ecp2* gene indeed encodes the ECP2 protein we purified and sequenced the N-terminal part of the protein. The sequence obtained (NH₂ - X A G X X P G S N R C D A S T F N N G Q - COOH) was in agreement with the amino acid sequence as deduced from the DNA sequence. The mature ECP2 protein, as

isolated from the extracellular space, is one N-terminal amino acid shorter than the processed protein, as predicted by the SigSeq program.

DISCUSSION

Apoplasmic fluid of *C. fulvum*-infected tomato leaves contains several low molecular weight proteins (<20 kDa) which are absent in culture filtrates of *in vitro*-grown *C. fulvum* and healthy tomato plants. The accumulation of these proteins in infected tomato plants is correlated with the increase in fungal biomass in the leaf, and these proteins were therefore thought to be of fungal origin (De Wit *et al.* 1989). Two of these proteins, ECP1 (synonymous with P1, Joosten and De Wit 1988) and ECP2 (J. P. Wubben, unpublished) were purified. Amino acid sequence data of the purified ECP1 enabled us to employ oligonucleotide probes to isolate the corresponding cDNA and gene (*ecp1*). Polyclonal antiserum raised against ECP2 was used to screen a cDNA expression library of infected tomato leaves, resulting in the isolation of the cDNA and gene (*ecp2*).

The *ecp1* gene encodes a precursor protein of 96 amino acids. The processed protein is 65 amino acids in size (7 kDa), as deduced from the DNA-sequence and N-terminal amino acid sequence of the purified protein from *C. fulvum*-infected tomato (Figs. 1 and 3). The original paper on the purification of ECP1 reports an estimated molecular weight of 14 kDa (Joosten and De Wit 1988), which is much higher than the deduced molecular weight of 7 kDa. By using the Tricine-SDS-PAGE system, the molecular weight was estimated to be 9 kDa, which is more in agreement with the deduced size of 7 kDa. The processing of the 96 amino acids precursor protein to the 65 amino acids mature protein most probably involves two steps. First, the protein is secreted and the signal peptide is cleaved off. The signal sequence cleavage site, as predicted by Von Heijne (1986) is between the amino acid residues Gly(23) and Gly(24) (Fig. 3). The second step probably takes place in the extracellular space and involves the activity of (plant or fungal) proteases on the N-terminal part of the secreted protein, resulting in a stable protein of 65 amino acids. This phenomenon has been observed for the race-specific peptide elicitor encoded by the avirulence gene *avr9* of *C. fulvum*, which is secreted in the extracellular space as a 40 amino acids peptide but is sequentially degraded to a very stable 28 amino acids peptide (G. F. J. M. Van den Ackerveken, P. Vossen, and P. J. G. M. De Wit, unpublished).

The processing of the ECP2 protein is similar to that of the ECP1 protein. The *ecp2*-encoded precursor protein of 165 amino acids is secreted into the extracellular space after cleavage of the signal peptide between residues Pro(22) and Arg(23) as predicted by Von Heijne (1986). Of the resulting 143 amino acids preprotein, amino acid residue Arg(23) is most probably removed by proteases present in the extracellular space, as the mature protein isolated from *C. fulvum*-infected tomato leaves starts with amino acid residue 24 (X A G). The estimated size of 17 kDa from SDS-PAGE (J. P. Wubben, unpublished) is in good agreement with the 142 amino acids protein as deduced from the DNA sequence.

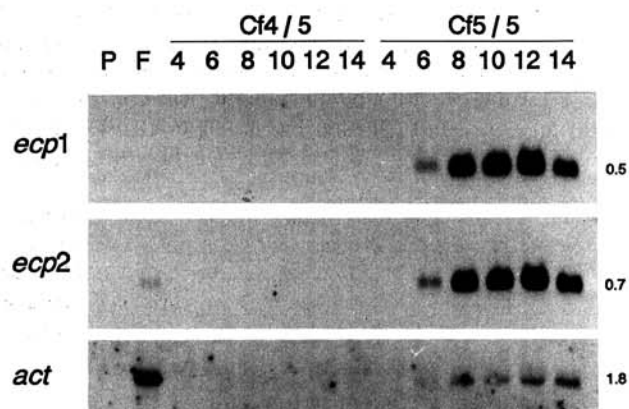


Fig. 5. Time course of accumulation of the *ecp1*, *ecp2*, and *act* mRNA. The cDNA inserts (*ecp* genes) and genomic subclone (*act*) were labeled by random priming and hybridized to a Northern blot containing equal amounts of poly(A)⁺-RNA isolated from uninoculated tomato genotype Cf5 (lane P), from *C. fulvum* grown *in vitro* (lane F), from an incompatible interaction Cf4/race 5 (Cf4/5) and a compatible interaction Cf5/ race 5 (Cf5/5) at different times (4, 6, 8, 10, 12, and 14 days, respectively) after inoculation. The sizes of the hybridizing mRNAs in kilobases is indicated.

The structure and organization of the two cloned *ecp* genes of *C. fulvum* is typical for filamentous fungi (Figs. 3 and 4). Motifs that are commonly found in genes of higher eukaryotes are also present in genes of filamentous fungi, but whether they are functional is not always clear (Gurr *et al.* 1988). The introns in the *ecp* genes are small, which is common for filamentous fungi. The splice junctions (GT..AG) and internal consensus sequence (TACTAAC) are present in all three reported introns. The two *ecp* genes both contain a possible TATA box and a CAAT box which might be involved in the regulation and positioning of transcription. The transcription start of the *ecp2* gene is located on the CAAG sequence, which is a common transcription initiation sequence in many yeast genes. The *ecp* genes both lack a typical polyadenylation signal (AATAAA) which is commonly found in genes of higher eukaryotes, but not frequently in genes of filamentous fungi (Gurr *et al.* 1988).

The *ecp* genes and the previously cloned avirulence gene *avr9* (Van Kan *et al.* 1991) are highly expressed *in planta* as compared to the *in vitro* situation. The mechanisms of induction or derepression for these genes are not known. The promoters of these induced genes do not reveal common structural motifs, which could be involved in the regulation *in planta*, although small homologies are present between *ecp1* and *ecp2* (data not shown). Future research using promoter-reporter gene fusions will enable us to define the promoter regions that are involved in the regulation of transcription.

The role of the *ecp* genes, which are functionally present in all races of *C. fulvum* tested, in establishing and maintaining basic compatibility remains unclear yet. The DNA sequences and derived amino acid sequences of the *ecp* genes have not given any indication of possible enzymatic or structural functions. The high expression of the *ecp* genes *in planta* as compared to *in vitro* and the abundance of the *ecp* proteins in the extracellular space of infected tomato leaves suggest a role in pathogenicity. The extracellular localization as described by J. P. Wubben (unpublished) suggests a role in the matrix which is present between the fungal hyphae and the host cell wall. Possibly, the *ecp* proteins are actively interfering with the metabolism and/or transport of host nutrients within the tomato leaf. Gene disruption by transformation and homologous recombination with mutated *ecp* genes is currently being carried out to determine whether these genes are essential for pathogenicity of *C. fulvum*.

MATERIALS AND METHODS

Subculture of *C. fulvum* and inoculation of tomato.

Cladosporium fulvum Cooke (syn. *Fulvia fulva* (Cooke) Cif) was grown on potato-dextrose agar (PDA) or in liquid B5 medium in shake cultures (De Wit and Flach 1979). Tomato genotypes containing different genes for resistance to *C. fulvum* were inoculated with a conidial suspension of *C. fulvum* containing 5×10^6 conidia ml⁻¹. Six- to seven-week-old plants were sprayed twice at the lower side of the leaf. The plants were allowed to dry and subsequently incubated at 100% humidity for 2 days to allow the spores to germinate (De Wit 1977). The infection of susceptible

tomato genotypes can be described as follows. The fungus penetrates the leaf through the stomata at 3–5 days after inoculation, followed by abundant fungal growth in the intercellular spaces of the leaf (days 4–11). Sporulation becomes visible at 12 days after inoculation, and becomes intense between 2–3 wk after inoculation (De Wit 1977; Lazarovits and Higgins 1976a).

Purification of compatible interaction specific proteins.

Intercellular fluid was isolated from the compatible tomato Cf5/*C. fulvum*, race 5 interaction at 14 days after inoculation. Protein ECP1 was purified by cation exchange chromatography, chromatofocusing, and gel filtration as described previously (Joosten and De Wit 1988) and the N-terminus was sequenced. Polyclonal antiserum, raised against protein ECP2, purified by gel filtration and anion exchange HPLC (J. P. Wubben, unpublished), was used to screen a cDNA expression library.

Screening of cDNA and genomic libraries.

A λZAP library containing cDNA synthesized on poly(A)⁺-RNA isolated from infected leaves of a compatible tomato Cf5/*C. fulvum*, race 5 interaction at 11 days after inoculation (Van Kan *et al.* 1992) was plated with *E. coli* strain PKLF' to obtain 100,000 plaques. Filters were hybridized with a [γ -³²P]ATP 5'-end-labeled oligonucleotide probe (Fig. 1, probe II). Positive phages were purified by replating and a second round of screening.

A λgt11 expression library containing cDNA synthesized on poly(A)⁺-RNA isolated from infected leaves of a compatible Cf5/race 5 interaction at 14 days after inoculation (Van Kan *et al.* 1991) was plated with *E. coli* strain Y1090 to obtain 250,000 plaques. Three hours after plating and incubation at 42° C, nitrocellulose filters (Schleicher & Schuell), soaked in 0.25% isopropyl-β-D-thiogalactopyranoside (IPTG) and dried, were placed on the top agar and incubated for 4 hr at 37° C. The filters were removed, blocked with 1% gelatine in TBST (Tris-buffered Saline, Tween), and subsequently incubated overnight with polyclonal antiserum raised against purified ECP2 (diluted 1:500 in TBST, 1% *E. coli* lysate, 0.5% bovine serum albumin, and 0.02% NaN₃). The antigen-antibody complexes were detected with the Bio-Rad Immuno-Blot goat anti-rabbit alkaline phosphatase (GAR-AP) assay kit. Positive phages were purified by replating and a second round of screening.

A λ EMBL3 genomic library of race 5 of *C. fulvum* was constructed and screened with the cDNA-clones obtained as described (Van den Ackerveken *et al.* 1992). The cDNA inserts were used as probes and were radioactively labeled with [³²P]dATP by the random primer method (Hodgson and Fisk 1987).

Cloning procedures and DNA sequencing.

All DNA manipulations were conducted essentially as described by Maniatis *et al.* (1982). DNA sequencing was performed using the chain termination method of Sanger *et al.* (1977).

RNA isolation, Northern blotting, primer extension, and RNA sequencing.

RNA was isolated from freeze-dried material as de-

scribed (Van Kan *et al.* 1991). Poly(A)⁺-RNA was obtained by affinity chromatography on oligo(dT)-cellulose, electrophoresed on denaturing formaldehyde-agarose gels, and blotted onto Hybond N membranes (Amersham) as described by Maniatis *et al.* (1982). The *C. fulvum* actin gene (*act*) was isolated from the genomic library using the *Phytophthora infestans actA* gene (Unkles *et al.* 1991) and used as a constitutive control for the Northern blot time course. RNA sequencing and primer extension was performed on poly(A)⁺ - RNA from a compatible *C. fulvum*-tomato interaction using 5'-end-labeled oligonucleotide primers as described (Van Kan *et al.* 1991).

Polyacrylamide gel electrophoresis (PAGE).

Sodium dodecyl sulphate (SDS)-PAGE on 15% (w/v) polyacrylamide slab gels was performed as described (Joosten and De Wit 1988). The molecular weight of protein ECP1 was determined on Tricine-SDS-PAGE gels (16.5%T, 6%C) as described by Schägger and Von Jagow (1987).

ACKNOWLEDGMENTS

These investigations were supported by the foundation for Biological Research (BION), which is subsidized by The Netherlands Organization for Scientific Research (NWO). We are grateful to Möisinger of Sandoz for sequencing the N-terminal part of protein ECP1, to MOGEN for synthesizing different oligonucleotide probes and to N. Danhash for critically reading the manuscript.

LITERATURE CITED

- De Wit, P. J. G. M. 1977. A light and scanning-electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum*. *Neth. J. Plant Pathol.* 83:109-122.
- De Wit, P. J. G. M., and Flach, W. 1979. Differential accumulation of phytoalexins in tomato leaves, but not in fruits after inoculation with virulent and avirulent races of *Cladosporium fulvum*. *Physiol. Plant Pathol.* 15:257-267.
- De Wit, P. J. G. M., Van den Ackerveken, G. F. J. M., Joosten, M. H. A. J., and Van Kan, J. A. L. 1989. Apoplastic proteins involved in communication between tomato and the fungal pathogen *Cladosporium fulvum*. Pages 273-280 in: *Signal Molecules in Plants and Plant-Microbe Interactions*. B. J. J. Lugtenberg, ed. Springer-Verlag, Berlin.
- Devereux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
- Gurr, S. J., Unkles, S. E., and Kinghorn, J. R. 1988. The structure and organization of nuclear genes of filamentous fungi. Pages 93-139 in: *Gene Structure in Eukaryotic Microbes*. J. R. Kinghorn, ed. IRL Press, Oxford.
- Hodgson, C. P., and Fisk, R. Z. 1987. Hybridization probe size control: Optimized 'oligolabeling'. *Nucleic Acids Res.* 15:6295.
- Joosten, M. H. A. J., and De Wit, P. J. G. M. 1988. Isolation, purification and preliminary characterization of a protein specific for compatible *Cladosporium fulvum* (syn. *Fulvia fulva*)-tomato interactions. *Physiol. Mol. Plant Pathol.* 33:241-253.
- Joosten, M. H. A. J., and De Wit, P. J. G. M. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3- β -glucanases and chitinases. *Plant Physiol.* 89:945-951.
- Joosten, M. H. A. J., Hendrickx, L. J. M., and De Wit, P. J. G. M. 1990. Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Neth. J. Plant Pathol.* 96:103-112.
- Lazarovits, G., and Higgins, V. J. 1976a. Histological comparison of *Cladosporium fulvum* race 1 on immune, resistant, and susceptible tomato varieties. *Can. J. Bot.* 54:224-234.
- Lazarovits, G., and Higgins, V. J. 1976b. Ultrastructure of susceptible, resistant, and immune reactions of tomato to races of *Cladosporium fulvum*. *Can. J. Bot.* 54:235-249.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Schägger, H., and Von Jagow, G. 1987. Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379.
- Unkles, S. E., Moon, R. P., Hawkins, A. R., Duncan, J. M., and Kinghorn, J. R. 1991. Actin in the oomycetous fungus *Phytophthora infestans* is the product of several genes. *Gene* 100:105-112.
- Van den Ackerveken, G. F. J. M., Van Kan, J. A. L., and De Wit, P. J. G. M. 1992. Molecular analysis of the avirulence gene *avr9* of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. *Plant J.* 2:359-366.
- Van Kan, J. A. L., Van den Ackerveken, G. F. J. M., and De Wit, P. J. G. M. 1991. Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mould. *Mol. Plant-Microbe Interact.* 4:52-59.
- Van Kan, J. A. L., Joosten, M. H. A. J., Sibbel-Wagemakers, C. A. M., Van den Berg-Velthuis, G. C. M., and De Wit, P. J. G. M. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. *Plant Mol. Biol.* 20:513-527.
- Von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683-4690.