The Biotrophic Fungus \textit{Cladosporium fulvum} Circumvents Cf-4-Mediated Resistance by Producing Unstable AVR4 Elicitors

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The avirulence gene \textit{Avr4} conditions avirulence of the biotrophic fungus \textit{Cladosporium fulvum} on tomato genotypes carrying resistance gene Cf-4 (MM-Cf4). Strains of the fungus that circumvent Cf-4-specific resistance show various single point mutations in the coding region of the \textit{Avr4} gene. Similar to expression of the \textit{Avr4} gene, expression of the various virulent \textit{avr4} alleles is specifically induced during pathogenesis. Polyclonal antibodies raised against the AVR4 elicitor, however, did not detect AVR4 isoforms in MM-Cf4 plants infected by the different virulent strains, indicating that these isoforms are unstable. To analyze whether the AVR4 isoforms still possess specific elicitor activity, the \textit{avr4} alleles were expressed in MM-Cf4 plants by using the potato virus X (PVX)-based expression system. Inoculation with PVX::Avr4 resulted in the development of spreading lesions, eventually leading to plant death, whereas the various PVX::avr4 derivatives induced symptoms ranging from severe necrosis to no lesions at all. We conclude that instability of the AVR4 isoforms that are produced by virulent strains is a crucial factor in circumvention of Cf-4-mediated resistance.

INTRODUCTION

The interaction between the biotrophic fungal pathogen \textit{Cladosporium fulvum} and its only host, tomato, is used as a model system to study the molecular basis of signaling events between a pathogen and its host (De Wit, 1995; Joosten et al., 1997). Colonization of the tomato leaf remains restricted to the intercellular spaces, and consequently, a large interface exists over which communication between plant and fungus takes place. Apoplastic fluid (AF), isolated from infected leaves after vacuum infiltration with water (De Wit and Spikman, 1982), contains various substances that are indicative of intense interchanges between \textit{C. fulvum} and tomato (De Wit et al., 1986; Joosten et al., 1990a).

The \textit{C. fulvum}–tomato interaction complies with the gene-for-gene model (De Wit, 1992); resistance of tomato is based on specific recognition of the invading fungus. Recognition activates a hypersensitive response in the host and is assumed to be the result of a direct interaction between receptorlike proteins, possibly encoded by resistance genes (Cf genes), and fungal race-specific elicitors encoded by avirulence genes (Avr genes). The induction of the hypersensitive response occurs specifically in an incompatible interaction between tomato and \textit{C. fulvum} and prevents colonization of the tomato leaves by the fungus. In the case of compatibility, no recognition of the fungus takes place, which eventually results in colonization of the tomato leaves. Jones et al. (1994) and Dixon et al. (1996) have cloned and characterized two genes conferring resistance of tomato to \textit{C. fulvum}. They are Cf-9 and Cf-2, and the DNA sequences of both genes predict that they encode plasma membrane–anchored extracytoplasmic proteins containing multiple leucine-rich repeat motifs that could be involved in protein–protein interactions (Kobe and Deisenhofer, 1994).

Two avirulence genes from \textit{C. fulvum}, \textit{Avr9} and \textit{Avr4}, have been cloned and characterized (Van den Ackerveken et al., 1992; Joosten et al., 1994). The \textit{Avr9} gene encodes a secreted peptide, the AVR9 elicitor, that is extremely protease resistant and consists in its mature form of 28 amino acids (Van Kan et al., 1991). The peptide has a rigid, barrel-shaped structure containing three antiparallel \(\beta\)-sheets connected by two loops and three disulfide bridges linking all six cysteine residues in a cystine knot (Vervoort et al., 1997). Strains of \textit{C. fulvum} that are virulent on tomato containing the Cf-9 resistance gene completely lack the \textit{Avr9} gene (Van Kan et al., 1991). Recently, it has been shown that there is a high-affinity binding site for the AVR9 elicitor present on
plasma membranes isolated from tomato leaves (Kooman-Gersmann et al., 1996). Besides tomato plants carrying the Cf-9 gene (MM-Cf9), tomato cultivar Moneymaker (MM-Cf0), which lacks all known genes for resistance against C. fulvum, and all solanaceous species analyzed so far also appear to contain such a binding site. Until now, the role of the CF-9 protein in AVR9 perception remains unclear.

Analysis of the open reading frame (ORF) of the Avr4 gene revealed that the primary translation product is a protein of 135 amino acids. After removal of the signal peptide for extracellular targeting, the protein is processed at the N terminus by plant and/or fungal proteases, resulting in a protein of 105 amino acids containing eight cysteine residues (Joosten et al., 1994). Instead of deletion of the gene, circumvention of Cf-4-mediated resistance was found to reside in single base pair changes in the ORF of the Avr4 gene. In earlier studies, the point mutations that were identified in virulent avr4 alleles all consisted of codon changes, resulting in substitution of a cysteine residue by a tyrosine residue (Joosten et al., 1994). It was suggested that loss of a cysteine residue, which might be involved in a disulfide bridge, affects the structure of the AVR4 protein in such a way that binding to the corresponding receptor is hampered, thus abolishing specific recognition of the fungus. The mutated protein would probably still be functional for the fungus itself.

To validate this hypothesis, we have performed a detailed analysis of additional strains of C. fulvum virulent on MM-Cf4 plants. In this study, we show that besides substitution of cysteine residues, other amino acid exchanges in the ORF of the Avr4 gene can also cause virulence in C. fulvum on tomato genotype MM-Cf4. Proteins homologous to the AVR4 elicitor that are encoded by the various avr4 alleles could not be detected, suggesting that these AVR4 isoforms are very unstable. However, expression of the avr4 alleles in MM-Cf4 plants by using the potato virus X (PVX) expression system revealed that some of the AVR4 isoforms appear to contain such a binding site. Until now, the role of the CF-9 protein in AVR9 perception remains unclear.

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RESULTS

Isolation and Characterization of a Genomic Avr4 Clone

To obtain information on the structure of the Avr4 gene, a genomic library of race 5 of C. fulvum (Van den Ackerveken et al., 1992) was probed with the Avr4 cDNA. Screening of ~50,000 recombinant plaques resulted in the identification of a 5.3-kb SstI fragment containing the complete ORF of the Avr4 gene and ~1.8 kb of the upstream region. This genomic Avr4 clone has been used to transform a strain of race 4 of C. fulvum to avirulence on MM-Cf4 plants (Joosten et al., 1994). Sequencing of overlapping subclones of the fragment resulted in the sequence presented in Figure 1. Alignment of the 5’ upstream region of the Avr4 gene with the promoter sequences of avirulence gene Avr9 (Van den Ackerveken et al., 1992) and the pathogenicity genes Ecp1 and Ecp2 of C. fulvum (encoding the extracellular proteins ECP1 and ECP2, respectively; Van den Ackerveken et al., 1993), which are also C. fulvum genes that are specifically expressed upon host colonization, did not reveal any significant homology.

Determination of the molecular mass of the AVR4 elicitor protein purified from the AF by electrospray mass spectrometry revealed that besides cleavage at the N terminus (Joosten et al., 1994), C-terminal processing of the AVR4 proprotein also takes place. Among the various intermediates that were identified, the most abundant form of the elicitor was found to correspond to an internal sequence of 86 amino acids (Figure 1).

Different Single Point Mutations Are Present in the avr4 Alleles of Strains of C. fulvum Virulent on MM-Cf4 Plants

Sequencing of the ORF of the avr4 allele present in strains of C. fulvum virulent on MM-Cf4 plants initially revealed that virulence resulted from replacement of a single cysteine residue by a tyrosine residue in the mature AVR4 protein (Joosten et al., 1994). As presented in Figure 2B, segment I, in these strains (isolated in the Netherlands, Poland, and France), the point mutation (a change from TGT to TAT) is present in the codon of Cys-64, Cys-70, or Cys-109. Recently, analysis of two additional virulent European isolates revealed that changes in codons for other amino acids can also cause circumvention of Cf-4-mediated resistance. One Dutch strain (4(2); race 4) was found to contain a nucleotide change in codon 67 (TAC to CAC) of the ORF of Avr4, resulting in a Tyr-to-His substitution, whereas analysis of a French strain (La Maxe 2; race 4) revealed a point mutation (ACC to ATC), replacing Thr-66 with Ile (Figure 2B, segment II). In another French isolate (Alénvya B; race 2.4.5), we identified a Cys-109→Tyr change, which is a mutation that has already been described (Joosten et al., 1994).

One strain from the United States is particularly interesting. In commercial greenhouses in Ohio, it appeared on tomato lines Purdue 38 and Purdue 135 (strain 38; race 4) (Bailey and Kerr, 1964). Instead of a nucleotide exchange, this strain shows a deletion of a single nucleotide, causing a frameshift in the ORF of the avr4 allele. From the stretch of four cytosine nucleotides spanning codons 42 (Pro) and 43 (Gln) (Figure 1), one nucleotide is deleted, resulting in an ORF encoding a truncated protein containing only 13 N-terminal amino acids of the mature AVR4 elicitor protein (Figure 2B, segment III). The ORF of the Avr4 gene of three
The gene encodes a precursor protein of 135 amino acids that is targeted to the extracellular space (the putative signal sequence is underlined) and processed at the N and C terminal. The sequence of the most abundant form of the mature AVR4 elicits by 86 amino acids is indicated in boldface type.

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**Expression Studies with Avirulence Gene Avr4 and Different Virulent Avr4 Alleles**

Previous experiments have shown that expression of the Avr4 gene of race 5 of *C. fulvum* is strongly induced when tomato leaves are colonized; by 6 days after inoculation and onward, Avr4 transcripts were detected on an RNA gel blot.

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**Figure 1. Nucleotide Sequence of Avirulence Gene Avr4 of *C. fulvum***

One-kilobase sequence of the upstream region, the ORF, and the 0.5-kb terminator region of the Avr4 gene are shown (EMBL accession number Y06356). The gene encodes a precursor protein of 135 amino acids that is targeted to the extracellular space (the putative signal sequence is underlined) and processed at the N and C termini. The sequence of the most abundant form of the mature AVR4 elicit of 86 amino acids is indicated in boldface type. Putative TATA and CAAT boxes in the promoter region are underlined. The coding region does not contain an intron sequence.

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**Figure 2. Diagram of the AVR4 Preproprotein and Its Isoforms from Different Strains of *C. fulvum* Virulent on MM-Cf4 Tomato.**

(A) The Avr4 preproprotein encoded by the putative gene Avr4 of strains avirulent on tomato genotype MM-Cf4.

(B) Strains virulent on MM-Cf4 plants carry an avr4 allele containing a single point mutation in the ORF. At the protein level, the mutations can be classified in three groups. Segment I indicates the substitution of the threonine residue at position 66 by an isoleucine residue or replacement of the tyrosine residue. Segment II indicates the substitution of the threonine residue at position 66 by an isoleucine residue or replacement of the tyrosine residue.
whereas only very low Avr4 expression levels were detected in the fungus grown in a liquid shake culture (Joosten et al., 1994; Van den Ackerveken and De Wit, 1994). Figures 3A to 3D show RNA gel blot analysis of total RNA isolated from compatible interactions involving different strains that contain the Avr4 gene (races 0, 2, 5, 2.5, or 2.5.9). Although considerable differences in expression levels occur, expression of the Avr4 gene in all strains is induced (Figure 3A).

Strains showing relatively high steady state Avr4 transcript levels also produced relatively high amounts of the AVR4 elicitor (races 5 and 2.5.9; results not shown), indicating that production of the elicitor is regulated at the transcriptional level. The low necrosis-inducing activity on MM-Cf4 plants that was found for AF isolated from the interaction between MM-Cf2 and race 2 (results not shown) appeared to be due to a less intense colonization of the leaves that were used for analysis, indicated by the low level of transcripts of the fungal actin gene (Figure 3B, lane 2). Detailed analysis of leaflets of MM-Cf4 plants inoculated with the various Avr4-containing strains (incompatible interaction) did not reveal differences in penetration rate or induction of host defense responses, such as callose deposition (Lazarovits and Higgins, 1976; De Wit, 1977) or accumulation of pathogenesis-related (PR) proteins (Joosten and De Wit, 1989), that could be related to the amount of AVR4 elicitor produced (results not shown). Probing of identical RNA gel blots with cDNA inserts of the Ecp1 or Ecp2 genes of *C. fulvum* (Van den Ackerveken et al., 1993) revealed that the steady state transcript levels of these genes do not in all cases correspond to the mRNA levels of the Avr4 gene (compare Figures 3C and 3D with Figure 3A), indicating that the three genes are not regulated similarly.

Gel blot analysis of total RNA isolated from MM-Cf4 plants colonized by various strains containing an avr4 allele revealed that although differences in expression levels were again observed, the avr4 allele is transcribed in all strains (Figure 3E; results not shown for the Cys64Tyr mutant). Considering the amount of fungal biomass present (indicated by the actin signal; Figure 3F), the avr4 transcript levels appear to be within the same range as those of the Avr4-expressing strains (compare Figures 3A and 3B with Figures 3E and 3F).

To detect at which stage of the infection process transcription of the Avr4 gene or its alleles occurs, we fused the promoter of the Avr4 gene to the β-glucuronidase (Gus) reporter gene and transferred this construct to *L. maculans* 2, a strain of *C. fulvum* virulent on tomato genotype MM-Cf4. Selected transformants were inoculated on 5-week-old MM-Cf4 plants, followed by histochemical localization of Gus activity. Figure 4A shows that immediately upon penetration of the leaf through the stomata, the Avr4 promoter is strongly activated. In the straight-growing runner hyphae present on the leaf surface, no Gus activity was detected. At later stages of infection, when abundant colonization of the entire leaf mesophyll has taken place, hyphae showing Gus activity are mainly localized in the vicinity of the vascular tissue (Figure 4B). The conidiophores that emerge from the stomata at ~10 to 14 days after inoculation show low Gus activity (Figure 4C). On resistant MM-Cf5 plants, activity of the Avr4 promoter can be detected clearly around the penetration of the leaf through the stomata, the Avr4 promoter is strongly activated. In the straight-growing runner hyphae present on the leaf surface, no Gus activity was detected. At later stages of infection, when abundant colonization of the entire leaf mesophyll has taken place, hyphae showing Gus activity are mainly localized in the vicinity of the vascular tissue (Figure 4B). The conidiophores that emerge from the stomata at ~10 to 14 days after inoculation show low Gus activity (Figure 4C). On resistant MM-Cf5 plants, activity of the Avr4 promoter can be detected clearly around the penetrated stomata. Because fungal growth remains restricted to a few mesophyll cells, staining for Gus activity results in the appearance of several blue-stained patches scattered over the leaf surface (Figure 4D).

![Figure 3. RNA Gel Blot Analysis of Total RNA Isolated from Compatible *C. fulvum*-Tomato Interactions Involving Strains That Either Contain the Avr4 Gene or One of the Different Virulent avr4 Alleles.](image)
Detection of AVR4 Isoforms

The observation that the different avr4 alleles of *C. fulvum* are highly transcribed during pathogenesis prompted us to determine whether proteins homologous to the AVR4 elicitor accumulate in the intercellular spaces of colonized leaves of MM-Cf4 plants. Strains containing the various avr4 alleles were inoculated onto MM-Cf4 plants, the AF was isolated from the colonized leaves after 14 to 20 days, and proteins were fractionated by gel filtration (Joosten et al., 1990b). The proteins of each AF sample were separated into seven fractions and analyzed for cross-reaction with polyclonal antibodies raised against the AVR4 elicitor. Immunoblot analysis revealed that none of the protein fractions reacted with the antiserum (results not shown).

Figures 5A and 5B summarize the analyses in which the AVR4-containing protein fraction of a strain expressing the Avr4 gene (race 5) was compared with similar gel filtration fractions of AFs originating from plants colonized by strains expressing the avr4 alleles. Although polyclonal antibodies raised against the pathogenicity factor ECP2 (Wubben et al., 1994) and the pathogenesis-related protein PR-4a (Joosten et al., 1990b) clearly detected a protein in all fractions, the AVR4 antibodies only identified the elicitor in the fraction originating from race 5 (results not shown for race 2.4.8.11 (Cys64Tyr)). Additional experiments involving further purification and concentration of the proteins present in the various gel filtration fractions by reversed-phase fast performance liquid chromatography (Joosten et al., 1994) or analysis of total leaf homogenate also did not result in detection of AVR4 isoforms (results not shown).

Expression of Avr4 and the Various avr4 Alleles in Tomato by PVX

The various avr4 alleles present in strains of *C. fulvum* virulent on tomato genotype MM-Cf4 are highly expressed in (incompatible interaction), and peels of the lower epidermis or leaf discs were obtained at 4, 7, and 14 days after inoculation and stained for Gus activity.

(A) An epidermal peel shows three stomata that have been penetrated at 4 days after inoculation. Straight-growing runner hyphae growing over the epidermis are not stained (thick arrows), whereas the penetrating hyphae are stained blue and thickened (thin arrows).

(B) Hyphae present in the vicinity of vascular tissue show high Gus expression (see arrows; leaf disc taken at 7 days after inoculation).

(C) The reemerging mycelium and conidiophores show relatively low Gus activity (see arrows; epidermal peel taken at 14 days after inoculation).

(D) Hyphae that penetrate stomata of leaflets of the resistant genotype MM-Cf5 also show Gus activity. Because growth is inhibited soon after penetration, only small blue-stained patches are visible (see arrows; leaf disc taken at 7 days after inoculation).
colonized leaves. However, AVR4 isoforms could not be detected, which suggests that they are unstable. To determine whether the AVR4 isoforms are still elicitor active, we expressed the various avr4 alleles in MM-Cf4 tomato by using an expression system based on PVX (Chapman et al., 1992). To ensure secretion of the encoded protein to the apoplast protected, which suggests that they are unstable. To determine of Nicotiana tabacum (Hammond-Kosack et al., 1994). The expression system based on PVX (Chapman et al., 1992). The modified ORFs were placed 3' of a duplication of the subgenomic coat protein promoter present in the PVX expression vector, and the PVX derivatives were inoculated on tomato.

In a quadratic check, 4-week-old tomato plants of genotype MM-Cf4 and MM-Cf9 were inoculated with either PVX::Avr4 or the PVX::Avr9 derivative that was constructed by Hammond-Kosack et al. (1995). By day 5 after inoculation, necrotic spots had appeared on the cotyledons and leaflets of the MM-Cf4 and MM-Cf9 plants that had been inoculated with PVX::Avr4 and PVX::Avr9, respectively (results not shown). These spots rapidly expanded, eventually leading to complete necrosis and collapse of the inoculated cotyledons and leaves within the next 24 hr. Figure 6A shows that over the subsequent 5 days, concomitant with the systemic infection by the PVX derivative, necrosis had spread and had reached the meristematic tissue of the shoot apex, resulting in plant death. On MM-Cf4 plants infected by PVX::Avr9 and MM-Cf9 plants infected by PVX::Avr4, a necrotic reaction was not observed, indicating that host genotype specificity is fully retained when the avirulence genes are expressed from modified PVX (Figure 6A). These plants had developed the typical systemic mosaic symptoms of a viral infection by ~7 days after inoculation.

Inoculation of 4-week-old MM-Cf4 plants with the various PVX::avr4 derivatives revealed that some of the AVR4 isoforms still possess specific necrosis-inducing activity. From the three different avr4 alleles having a cysteine residue replaced by a tyrosine residue in the encoded AVR4 isoform, inoculation with PVX::avr4(Cys70Tyr) did not result in any epinasty or necrosis, whereas PVX::avr4(Cys64Tyr) and PVX::avr4(Cys109Tyr) caused clear AVR4-specific responses on MM-Cf4 plants, consisting of stunting, epinasty, and development of necrotic lesions on the leaflets (Figure 6B). Inoculation with PVX::avr4(Cys109Tyr) resulted in symptoms that closely resembled those caused by PVX::Avr4, although complete death of the plants did not occur. Inoculation with PVX::avr4(Tyr67His) did not induce any necrotic response (Figure 6B). Inoculation of MM-Cf4 plants with PVX containing the avr4 allele carrying the frameshift only resulted in the development of systemic mosaic symptoms. Inoculation of MM-Cf9 plants with the various PVX::avr4 derivatives also caused systemic mosaic symptoms only; in all cases, no necrosis or epinasty was observed (results not shown).

At 10 days after inoculation, the AF was isolated from the systemically infected MM-Cf9 plants and checked for the presence of the AVR4 protein or its isoforms by immunoblot analysis. The polyclonal AVR4 antibodies detected low amounts of the AVR4 elicitor only in the AF isolated from MM-Cf9 plants infected by PVX::Avr4 (results not shown). Also, when N. clevelandii or N. benthamiana plants were inoculated with the PVX derivatives, only in the AF isolated from plants that were infected by PVX::Avr4 could the AVR4 elicitor be detected in very low amounts, whereas none of the isoforms was detected (results not shown). RNA gel blot analysis of total RNA isolated from PVX-infected MM-Cf9 plants, using the coat protein gene as a probe, revealed that the subgenomic mRNA from which the coat protein is produced and the genomic RNA are highly abundant. However, the two subgenomic RNA molecules from which the PVX movement proteins and the AVR4 protein are produced

Figure 5. Detection of AVR4 Isoforms.
Shown are the results after SDS-PAGE and gel blot analysis of fractions obtained upon gel filtration of proteins present in the AF isolated from the compatible interaction between MM-Cf5 tomato and race 5 (a strain of C. fulvum that carries the Avr4 gene, lanes 1) and the compatible interactions between MM-Cf4 tomato and race 2.4.5 (Cys70Tyr, lanes 2), race 2.4 (Cys109Tyr, lanes 3), strain 38 (race 4, frameshift, lanes 4), strain 42 (race 4, Tyr67His, lanes 5), and strain La Maxe 2 (race 4, Thr66Ile, lanes 6). Approximately 25 μg of protein present in the fifth fraction of each gel filtration run from the different AFS was electrophoresed to obtain four replicate gels. Lane m contains molecular mass markers given in kilodaltons.
(A) SDS gel of proteins present in G-50 fractions stained with Coo- massie Brilliant Blue R 250. The arrows indicate the position of the ECP2, PR-4a, and AVR4 proteins.
(B) Immunoblots of three replicates of the gel presented in (A) incubated with polyclonal antibodies raised against ECP2 (Wubben et al., 1994), PR-4a (Joosten et al., 1990b), or AVR4.
Recognition and Stability of AVR4 Isoforms

were only present in very low amounts, indicating that the systemic expression level of the ORF that is introduced in the PVX vector is very low (results not shown).

DISCUSSION

Expression of the Avr4 Gene and Its Virulent Alleles

In all strains of C. fulvum that were analyzed, the Avr4 gene or its virulent avr4 allele is expressed. When the various strains that produce the AVR4 elicitor were compared, considerable differences in Avr4 mRNA levels (Figure 2) and in the amounts of AVR4 elicitor produced in colonized leaves were found. When resistant MM-Cf4 plants were inoculated, no differences in penetration rate or induction of host defense responses were found, implying that the amount of AVR4 elicitor secreted by all of these strains is above the minimum threshold level to induce resistance. These strains, which were collected in Europe, are highly heterogeneous regarding traits such as growth rate in vitro, sporulation rate, and color or colonization density of the host. The differences in Avr4 transcript levels probably are a reflection of this heterogeneity. Heterogeneity of the various strains that are virulent on MM-Cf4 tomato may also account for the observed differences in avr4 transcript levels. For these strains, however, differences in stability and translation efficiency of the various types of avr4 transcripts might also influence the steady state transcript levels.

Although the promoter regions of the Avr4 and Avr9 genes do not show any significant homology, analysis of transformants of C. fulvum containing an Avr4 or Avr9 promoter–Gus fusion indicated that the localization and timing of expression of both avirulence genes are highly similar. Immediately upon passage of the stomata, the genes are transcribed, whereas expression appeared to be the highest in mycelium growing in close contact with the vascular tissue (Figures 5A and 5B; Van den Ackerveken et al., 1994). The Avr9 promoter contains several nitrogen-responsive elements homologous to those found in Neurospora crassa (TAGATA and GATA boxes; Fu and Marzluf, 1990). For the induction of Avr9 gene expression, it was suggested that the absolute nitrogen concentration in the vicinity of the vascular bundles is constantly limiting because of the high metabolic activity of the fungus in this region (Van den Ackerveken et al., 1994). The 5' upstream region of Vne Avr4 gene does not contain these elements, indicating that expression of this gene is regulated in a different way. Expression studies with genes of C. fulvum that are specifically induced during pathogenesis suggest that Avr4 and Avr9 are among the first genes to be activated in the infection process. Combined with the relatively high stability of AVR4 and AVR9 in the apoplast, these proteins are primary candidates for the host to be recognized, rendering them avirulence factors of the fungus.
Production, Stability, and Elicitor Activity of AVR4 Isoforms

The various single nucleotide changes that have been found in the ORF of the different avr4 alleles appear to result in amino acid substitutions that affect the stability of the encoded AVR4 isoforms in the apoplast. In this respect, the single point mutations in the ORF of the Avr4 gene have an effect that is similar to the one resulting from complete absence of the encoding gene, as is the case for the Avr9 gene in strains of C. fulvum virulent on MM-Cf9 plants. Possibly, the chromosomal localization of the Avr4 gene does not allow severe alterations in the genomic structure. Results obtained by pulsed-field gel analysis of the chromosomes of C. fulvum indicated that Avr9 might be present on a part of the chromosome that is unstable and easily deleted in strains that are virulent on MM-Cf9 tomato (Talbot et al., 1991).

A similar phenomenon has been described for avirulence genes PWL2 (for pathogenicity toward weeping lovegrass) and AVR2-YAMO (for avirulence on the rice cultivar Yashiro-mochi) of the rice blast fungus Magnaporthe grisea (Valent and Chumley, 1994; Sweigard et al., 1995). From the PWL2 gene, three homologs have been cloned (Kang et al., 1995). One homolog (PWL1) was found to be functional, whereas PWL3 and PWL4 were nonfunctional as host specificity genes. In the case of PWL3, the situation is similar to that of the virulent avr4 alleles of C. fulvum; the ORF of this gene does not encode a protein that confers avirulence toward weeping lovegrass. For the PWL4 homolog, it was found that lack of function was due to improper expression.

The strain of C. fulvum containing the frameshifted avr4 allele sporulates abundantly on susceptible tomato plants, indicating that the Avr4 protein itself is not essential for fungal pathogenicity and that the functional Avr4 gene can be sacrificed to broaden the range of tomato genotypes that can be infected.

By using the PVX expression system, we have shown that at least three of the avr4 alleles encode an AVR4 isoform that is still elicitor active. Because the tertiary structure of these isoforms still allows specific recognition by the host, we assume that in this case, folding is not severely hampered, and therefore, polyclonal antibodies raised against the AVR4 elicitor are expected to cross-react with the AVR4 isoforms. We have, however, not been able to detect AVR4 isoforms in the AF from C. fulvum-infected MM-Cf9 plants.

The Nip1 gene (encoding necrosis-inducing peptide 1) of Rhynchosporium secalis, the causal agent of leaf scald on barley, encodes a virulence factor that acts as an elicitor of PR protein accumulation in barley plants that carry the Rrs-1 resistance gene. Strains of the fungus that are able to circumvent Rrs-1-mediated resistance were found either to lack the Nip1 gene or to contain a nip1 allele showing various nucleotide changes (Rohe et al., 1995). In contrast to the avr4 alleles of C. fulvum, the virulent nip1 alleles encode stable NIP1 isoforms. Similar to the Nip1 gene, the nip1 alleles are expressed in vitro, and the encoded isoforms are normally secreted. The isoforms do not induce PR proteins, indicating that the tertiary structure of the proteins is changed in such a way that they are not recognized by the Rrs-1-containing barley cultivars or that the protein has lost its stability in the plant. Furthermore, amino acid changes were found in the Nip1 gene that reduce the elicitor activity of the encoded NIP1 isoform but do not result in virulence on the Rrs-1 genotype. In the eight strains of C. fulvum avirulent on tomato genotype MM-Cf4 that we have analyzed, no differences in the sequence of the ORF of the Avr4 gene were found.

The single amino acid changes present in the AVR4 isoforms might affect correct folding of the proteins upon synthesis and thereby hamper secretion to the apoplast (MacKenzie et al., 1993; Peberdy, 1994; Schatz and Dobberstein, 1996). In C. fulvum-infected MM-Cf4 plants, however, an AVR4 isoform was also not detected when total leaf homogenates were analyzed. In our studies using various PVX::avr4 derivatives, we found that secretion of the AVR4 isoforms takes place when the avr4 alleles are expressed in tomato. These findings indicate that at least some of the AVR4 isoforms are properly secreted by C. fulvum during colonization of the tomato leaves. We suggest that when AVR4 isoforms are secreted, they are immediately degraded by plant and/or fungal proteases, thereby preventing binding to the complementary receptor.

Fate of AVR4 and Its Isoforms after Secretion by C. fulvum or by PVX-Infected Host Cells

Similar to the results obtained by Hammond-Kosack et al. (1995), who analyzed N. clevelandii plants that were systemically infected by PVX::Avr9, the abundance of the additional mRNA encoding the AVR4 elicitor or its isoforms in MM-Cf9 tomato infected by the various PVX derivatives was found to be very low. Immunoblot analysis indicated that the concentration of the AVR4 elicitor in AF isolated from PVX::Avr4-infected MM-Cf9 plants is much lower than in the AF isolated from tomato leaves colonized by strains of C. fulvum that carry the Avr4 gene. In the AF isolated from MM-Cf9 plants infected by the various PVX::avr4 derivatives, AVR4 isoforms could not be detected.

When tomato leaves either colonized by C. fulvum or systemically infected by the PVX derivatives are compared, the difference in the site of production of the AVR4 elicitor or its isoforms appears to be essential. In C. fulvum–infected tomato, the AVR4 protein is synthesized by the fungus, which is present in the intercellular spaces. Figure 7A illustrates that when the protein is secreted, it must pass cell walls of both the fungus and the plant to reach the complementary receptor, which is assumed to be located on the plasma membrane of the host cells (Kooman-Gersmann et al., 1996). The passage through the apoplast makes the AVR4 protein prone to degradation by extracellular host and fungal proteases, which are abundantly present. For example,
one of the major PR proteins that is induced in infected tomato leaves is an alkaline endoprotease (Vera and Conejero, 1988). Furthermore, production of extracellular proteases by *C. fulvum* appeared to be important for its pathogenicity (Kenyon, 1990). Increased susceptibility of the AVR4 protein to proteolytic breakdown due to changes in the primary structure prevents the protein from reaching the receptor, allowing the fungus to colonize the tomato leaves without induction of early host defense responses. In mesophyll cells of tomato leaves that are infected by PVX derivatives, the virus replicates and drives production of the AVR4 protein (Figure 7B). The protein is directly secreted from the host cells and does not have to pass cell walls of either plant or fungus to come into contact with its receptor, leaving less opportunity for degradation. In this way, even from AVR4 isoforms that are highly unstable, specific elicitor activity can be detected as long as their structure allows recognition by the host.

The observation that two of the AVR4 isoforms that contain a Cys-to-Tyr mutation still possess specific elicitor activity indicates that not all disulfide bridges in the protein are essential for recognition by the host. However, although these disulfide bridges do not appear to be crucial for maintaining the right conformation of the protein, the bonds are probably very important for stability of the protein in the intercellular spaces, a phenomenon commonly observed for secreted proteins (Peberdy, 1994). Although the Avr4 and Avr9 genes of *C. fulvum* are highly expressed in colonized tomato leaves, the amount of AVR4 elicitor that is present in the AF is relatively low when compared with the amount of AVR9 peptide present, indicating that the AVR4 protein is less stable. Single amino acid changes further decrease the stability of the secreted AVR4 isoforms, resulting in virulence on MM-Cf4 tomato of strains that produce these isoforms. Inoculation of tomato genotype MM-Cf4 with PVX::avr4 (Cys70Tyr) or PVX::avr4(Thr66Ile) did not result in any necrotic responses. These *avr4* alleles might encode either AVR4 isoforms that are extremely unstable or isoforms that are not able to fold correctly.

Further mutational analysis of the ORF of the *Avr4* gene, consisting of either single codon changes or deletions of parts of the ORF, might reveal which domains in the AVR4 protein are important for specific recognition by MM-Cf4 tomato. Detailed information on the mechanism by which the AVR4 elicitor interacts with its complementary receptor will provide more insight into the molecular basis of the recognition event that eventually leads to the activation of host defense responses and resistance.

**METHODS**

**Plant and Fungal Materials**

The near isogenic lines of tomato (*Lycopersicon esculentum*) cultivar Moneymaker, carrying the different genes for resistance against *Cladosporium fulvum* (syn *Fulvia fulva*), were grown as described by De Wit and Flach (1979). They are referred to as genotype MM-Cf0 (no known resistance genes), MM-Cf2, MM-Cf4, MM-Cf5, and MM-Cf9, corresponding to the *Rf* resistance gene that each carries. The race of a strain of *C. fulvum* is determined by the tomato resistance genes that the strain can overcome. For the resistance genes that cannot be overcome by this strain, the strain contains the corresponding avirulence genes. *Nicotiana clevelandii* and *N. benthamiana* were grown under the same conditions as the tomato plants. To obtain conidia for inoculation of tomato, *C. fulvum* was grown on solid
potato dextrose agar (De Wit and Flach, 1979). For DNA isolation, the fungus was grown on liquid B5 medium in shake culture (De Wit and Roseboom, 1980).

DNA and RNA isolation and DNA and RNA Gel Blotting

For DNA gel blot analysis, mycelium of C. fulvum was freeze dried, and DNA was isolated according to the method described by Van Kan et al. (1991) and digested with either Xhol or PstI. Ten micrograms of DNA was electrohoresed and blotted to a Hybond N+ membrane (Amersham International) by standard procedures. For polymerase chain reaction (PCR) analysis of genomic DNA of C. fulvum, DNA was isolated from mycelium grown in vitro or from infected tomato leaflets via a mini-preparation procedure, based on the method described by Lassner et al. (1989). RNA from C. fulvum–or potato virus X (PVX)–infected tomato leaves was isolated using the Extract-A-Plant RNA isolation kit (Clontech, Palo Alto, CA). RNA (15 μg isolated from C. fulvum–infected leaves or 3 μg of RNA from PVX–infected tomato leaves) was electrophoresed through gels containing formaldehyde, according to the method described by Sambrook et al. (1989), and blotted to a Hybond N+ membrane. DNA and RNA gel blots were prehybridized at 65°C in 0.5 M phosphate buffer, pH 7.2, containing 7% SDS and 1 mM EDTA (adapted from Church and Gilbert, 1984) and probed in the same buffer with cDNA or genomic fragments that were 32P-label by using the random primers DNA labeling system (Gibco BRL, Breda, The Netherlands) or the Ready- To-Go DNA labeling kit (Pharmacia Biotech, Roosendaal, The Netherlands). The blots were washed with 0.5 × SSC (75 mM NaCl, 7.5 mM sodium citrate) containing 0.5% SDS at 65°C.

Screening of a Genomic Library of C. fulvum

A genomic library of race 5 of C. fulvum constructed by Van den Ackerveken et al. (1992) was screened with a 32P-labeled cDNA insert encoding AVR4 according to the method described by Van den Ackerveken et al. (1992).

Sequencing of the Open Reading Frame of the avr4 Alleles

PCRs with DNA isolated from the various strains of C. fulvum or from C. fulvum–infected tomato leaflets were used to amplify the open reading frame (ORF) present in the Avr4 gene or its virulent alleles. PCR sequencing of the generated fragments was then performed as described previously by Joosten et al. (1994).

Fusion of the Promoter of Avr4 to the Reporter Gene Encoding β-Glucuronidase, Transformation of C. fulvum, and Selection and Histochemical Localization of β-Glucuronidase Activity

To obtain a perfect fusion between the Avr4 promoter and the uidA gene of Escherichia coli encoding β-glucuronidase (Gus), PCR was performed on 100 ng of plasmid DNA containing a genomic clone of Avr4. The T7 promoter primer (Promega, Leiden, The Netherlands) specific to the vector and a primer specific to the 3′ end of the promoter (5′-CGGGATCCATGGCTGAGTCTGAGTCTTGAGTCTTGAGTCTTGAGTCTTGAGTCTTGAGTCTTGAGTCTTGAGTCT) were used to obtain an Avr4 promoter fragment containing an Ncol site at the 3′ end. PCR (1 min at 95°C, 1 min at 55°C, and 1 min at 72°C for 30 cycles) was performed with Ultra DNA polymerase (Perkin-Elmer, Gouda, The Netherlands), according to the manufacturer’s protocol. The generated blunt-ended fragment of 1700 bp was ligated into EcoRV-digested pGEM-3Zf(+) (Promega) and transformed to E. coli DH5α. The recombinant plasmid was isolated and digested with EcoRI (present at 200 bp from the 5′ end of the promoter fragment) and Ncol. The resulting fragment was used to replace the glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter of Aspergillus nidulans in pCF20, which contains the gpd promoter fused to the uidA gene (Van den Ackerveken et al., 1994).

Strain La Maxe 2 (race 4, Thr6611) of C. fulvum was transformed with the construct containing the Avr4 promoter–Gus fusion, according to the method described by Oliver et al. (1987) and Harling et al. (1988), applying the modifications made by Van den Ackerveken et al. (1992). Twenty-three hygromycin-resistant transformants were inoculated on seedlings of MM-Cf4 plants, and of those showing clear induced Gus activity, eight transformants were selected to be analyzed in detail on 5-week-old MM-Cf4 and MM-Cf5 plants. Histochemical localization of Gus expression in leaf discs and peelings from the lower epidermis of tomato leaflets was performed as described by Van den Ackerveken et al. (1994).

Protein Fractionation and Detection of AVR4 and Its Isoforms

Isolation of apoplastic fluids (AFs) from the intercellular spaces of C. fulvum–infected tomato leaves was performed as described by De Wit and Spikman (1982). Total leaf homogenate was prepared as described by Van Kan et al. (1992). Gel filtration of proteins using Sephacryx G-50 (Pharmacia Biotech) was performed as described by Joosten et al. (1990b), and reversed-phase fast protein liquid chromatography on a ProRPC HR5/10 column (Pharmacia Biotech) was performed according to Joosten et al. (1994). SDS-PAGE and protein gel blot analysis were performed as described by Joosten and De Wit (1988) and Joosten et al. (1990b). Polyclonal antibodies were raised against the AVR4 elicitor by two subcutaneous injections of a mouse, each with 15 μg of the purified AVR4 protein, at an interval of 2 weeks. For the first injection, Freund’s complete adjuvant was used, whereas the booster was injected with incomplete adjuvant. The mouse was bled 2 weeks after the last injection.

Mass Determination of AVR4

The molecular mass of the mature AVR4 elicitor protein that was purified from the AF isolated from a compatible interaction involving race 5 of C. fulvum was determined by electrospray mass spectrometry. Spectra were collected during constant infusion of the sample with a syringe pump (model 2400; Harvard Apparatus, South Natick, MA) in a Finnigan MAT 900 (Finnigan MAT, San Jose, CA) equipped with a Finnigan MAT API ion source. The protein (5 μg) was dissolved in methanol–water (80/20 [v/v]) containing 1% (v/v) acetic acid and infused with a liquid flow rate of 1 μL per min.

Construction of PVX Derivatives and Transcription

To obtain functional expression of the Avr4 gene by using the expression system based on PVX (Chapman et al., 1992), a PVX::Avr4 derivative was constructed. For extracellular targeting of the AVR4 protein in the plant, the sequence encoding the AVR4 elicitor of 117 amino acids (the protein remaining after removal of the signal sequence) was fused to the 3′ end of the sequence encoding the sig-
nual peptide of the tobacco pathogenesis-related protein PR-1a (Cornelissen et al., 1987; Hammond-Kosack et al., 1995). For the fusion, the PCR overlap extension protocol of Horton and Pease (1991) was followed, using Ultra DNA polymerase (Perkin-Elmer).

The fragment containing the sequence encoding the AVR4 elicitor of 117 amino acids was obtained by performing PCR with a genomic clone of Avr4. At the 3' end of the ORF, a primer was used that introduces a Clal restriction site at the stop codon (5'-GGCTCTAT-CAGAGCTATCCATGATGAGCAAC-3' [AVR4CLA]), whereas at the 5' end of the ORF of the Avr4 gene, a primer containing an overhang of 11 nucleotides complementary to the 3' end of the sequence encoding the PR-1a signal peptide was used (5'-CTT-GCCGTGCCCAACCAATATCTCTTGCG-3' [AVR4III]). We amplified the fragment containing the sequence encoding the PR-1a signal peptide from the PVX::Avr4 construct (Hammond-Kosack et al., 1995) by using a primer specific to the 3' end of the PR-1a signal sequence containing an overhang of 14 nucleotides complementary to the 5' end of the ORF encoding AVR4 without signal sequence (5'-GGA-TTTGTTGGGCAACGGGAGATGGGATATAC-3') and a primer specific to the PVX-vector DNA flanking the 5' end of the PR-1a signal sequence (5'-CAATCAGTGTGGCTTGCC-3' [OX10]). Both PCRs were performed with 500 ng of plasmid DNA with the following protocol: 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C for 25 cycles.

The two fragments were run on a gel, purified, mixed (50 ng each), and amplified with OX10 and AVR4CLA-primers, using the same profile with an annealing temperature of 47°C. The amplified fragment, containing the PR-1a signal sequence fused to the ORF of the Avr4 gene, was digested with Clal, purified from the gel, and ligated into the Clal site of the PVX expression vector pTXAGC3A (Baulcombe et al., 1993). Transformants of E. coli DH5α containing the PVX vector with the PR-1a-Avr4 insert in the sense orientation were selected by PCR screening using a primer specific to the sequence encoding the signal peptide) was amplified from genomic DNA isolated from the different strains of C. fulvum virulent on genotypes MM-C14 by using the AVR4CLA and AVR4II-primers. PCRs were performed with 500 ng of genomic DNA isolated from races 2.4.8.11 (Cys64Tyr), 2.4.5 (Cys70Tyr), and 2.4.9.11 (Cys109Tyr) and strains La Maxe 2 (race 4, Thr6611e), 42 (race 4, Tyr67His), and 38 (race 4, frameshift). After verification of the nucleotide sequence by DNA sequencing, infectious transcripts were obtained in vitro from 0.5 µg of SstI-linearized recombinant PVX plasmid using the T7 mMessage mMachine transcription kit (Ambion, Austin, TX).

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### PVX Inoculations

The inoculations that were obtained from the various PVX plasmids were applied to 3- to 4-week-old N. clevelandii plants, and 7 days after inoculation, leaves showing systemic mosaic symptoms were homogenized in 50 mM sodium phosphate buffer, pH 7.0 (1 g fresh weight per mL of buffer). The homogenate was directly used to inoculate the cotyledons and top leaflets of the first and second compound leaves of 4-week-old tomato plants. From 4-week-old N. clevelandii and N. benthamiana plants, four of the lower leaves were inoculated.


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Recognition and Stability of AVR4 Isoforms


The biotrophic fungus Cladosporium fulvum circumvents Cf-4-mediated resistance by producing unstable AVR4 elicitors.

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