Molecular aspects of avirulence and pathogenicity of the tomato pathogen *Cladosporium fulvum*

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Hoogleraar in de Fytopathologie,
in het bijzonder de fysiologische aspecten

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Molecular aspects of avirulence and pathogenicity of the tomato pathogen *Cladosporium fulvum*

Proefschrift

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Subject headings: avirulence genes ; Cladosporium fulvum / pathogenicity genes ; Cladosporium fulvum.

Stellingen

1. Fysio-specifieke resistentie in tomaat tegen *Cladosporium fulvum* wordt geïnduceerd door één specifiek peptide van het pathogeen.
Dit proefschrift

2. Zolang Vanderplank specifieke herkenning van pathogenen in resistente planten niet erkent, kan het hem niet kwalijk worden genomen dat hij de "avirulentie versie" van de gen-om-gen hypothese absurd vindt.
Vanderplank (1991) Plant Pathology 40, 1-3

3. Het is voorbarig de verschillen in afweerreakties van planten tegen pathogenen toe te schrijven aan verschillen in resistentiegenen wanneer de vorming van de betrokken elicitoren niet nader onderzocht is.
Hammond-Kosack *et al.* (1993)
In: Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 2 :457-461

4. De stelling dat gen-om-gen interacties tussen plant en pathogeen enkel zijn ontstaan door de veredeling van cultuurgewassen middels stapsgewijze introductie van resistentiegenen is ongegrond, daar in natuurlijke plant-pathogeen populaties wel degelijk sprake is van gen-om-gen interacties.
Barrett (1985) In: Ecology and Genetics of Host-Parasite Interactions: 215-225
Crute *et al.* (1993) In: Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 2: 437-444

5. Het aantal plant-pathogeen interacties waarin fysio-specifieke elicitoren zijn aangetoond is waarschijnlijk zo gering omdat deze elicitoren niet abundant of (water-) oplosbaar zijn.

6. Invertase is voor *Cladosporium fulvum* niet alleen essentieel voor de benutting van sucrose, maar is mogelijk ook een pathogeniteitsfactor die floeembelading remt.
Dickinson *et al.* (1991) Plant Physiology 95, 420-425

7. De specificiteit van verwante bacteriële avirulentiegenen uit de *avrBs3* familie voor zowel dicotyle als monocotyle waardplanten maakt het aannemelijk dat de corresponderende resistentiegenen in deze planten ook nauw verwant zijn.
Bonas *et al.* (1993) Molecular and General Genetics 238, 261-269

8. Het effect van de CO_2-toename in de atmosfeer op de opwarming van de aarde is gering in vergelijking tot de verhitting van de discussies over dit onderwerp.

9. Het streven naar de instandhouding van heidegebieden in Nederland is eerder een zaak van cultuurbehoud dan van natuurbehoud.

10. Een wetenschappelijk onderzoeker is meer gebaat bij een opgeruimde geest dan bij een opgeruimd laboratorium.

11. Het onderscheid tussen "to play squash" en "to squash" is voor sommige spelers van deze racketsport niet geheel duidelijk.

Stellingen behorend bij het proefschrift "Molecular aspects of avirulence and pathogenicity of the tomato pathogen *Cladosporium fulvum*"

Wageningen, 12 november 1993 Guido van den Ackerveken

Voorwoord

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Dank aan stichting BION en het LEB-fonds voor hun bijdrage in de drukkosten van het proefschrift.

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Contents

List of abbreviations

aa	amino acid(s)
act	gene encoding actin
avr	avirulence gene
AVR	*avr* gene product
bp	base pair(s)
BSA	bovine serum albumin
Cf	gene for resistance to *Cladosporium fulvum*
ecp	gene encoding extracellular protein
ECP	*ecp* gene product, extracellular protein
EDTA	ethylene-diaminetetraacetic acid
FOA	5-fluoroorotic acid
gpd	gene encoding glyceraldehyde-3-phosphate dehydrogenase
GUS	ß-glucuronidase
hph	Hygromycin B resistance gene
HR	hypersensitive response
HRLC	high resolution liquid chromatography
HygR	hygromycin B resistant
IF	intercellular fluid
kb	kilo base (1000 bp)
kD	kilodalton
Mes	2-(*N*-morpholino)-ethanesulphonic acid
MM	tomato cultivar Moneymaker
Mr	relative molecular weight
nt	nucleotide(s)
OMPdecase	orotidine-5-phosphate decarboxylase
OPRTase	orotate pyrophosphoribosyl transferase
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PBS	phosphate buffered saline
PR	pathogenesis related
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
Tricine	*N*-tris(hydroxymethyl)-methylglycine
uidA	gene encoding ß-glucuronidase
UV	ultra violet light

Outline of this thesis

New strategies for crop protection are required for efficient and environmentally friendly agriculture. Durable resistance of plants to pathogens is the most effective means of crop protection, but can often not be achieved by traditional plant breeding. Molecular breeding for resistance is a new and alternative strategy for future crop protection. This new strategy can only be developed when more basic knowledge on plant-microbe interactions becomes available. As P.R. Day once formulated: 'Wise and intelligent methods of crop protection can only be based on an appreciation of the principles of host-parasite interaction and the consequences of interfering with it' (Day, 1974). Fundamental research on molecular plant-microbe interactions can reveal the mechanisms involved in pathogenicity and avirulence of pathogens. The activation of plant defence upon pathogen attack is mediated by an early recognition of the pathogen and a quick and adequate response by the resistant plant. Basic knowledge on pathogen recognition and induction of plant defence can eventually lead to the isolation and characterization of plant resistance genes. Molecular understanding of the structure and function of disease resistance genes could provide the basis for further development of molecular resistance breeding.

At the beginning of the PhD project described in this thesis research on the *Cladosporium fulvum* - tomato interaction was focused on physiological and biochemical aspects (Joosten, 1991). Plant response molecules such as phytoalexins (De Wit and Flach, 1979) and pathogenesis related proteins (Joosten and De Wit, 1989; De Wit *et al.*, 1989) were isolated and characterized. Compatible specific proteins were purified from the intercellular fluid of *C. fulvum* -infected tomato leaves (De Wit *et al.*, 1989). These extracellular proteins were suggested to be of fungal origin (Joosten and De Wit, 1988). The abundance of these proteins in *C. fulvum*-infected tomato leaves and their absence in *C. fulvum* grown *in vitro* suggested a possible role in pathogenesis.

The discovery of race-specific peptide elicitors of *C. fulvum* (De Wit and Spikman, 1982) and the subsequent purification and characterization of the AVR9 elicitor (Scholtens-Toma and De Wit, 1988) provided a solid basis for molecular studies on avirulence. Avirulence of races of *C. fulvum* on tomato genotypes carrying resistance gene *Cf9* was always correlated with the presence of the necrosis-inducing peptide elicitor in susceptible plants infected with these same races. The hypothesis was put forward that the elicitor originated from an avirulence gene present in races of *C. fulvum* avirulent on tomato genotype *Cf9*.

In this thesis research on the molecular aspects of avirulence and pathogenicity of the tomato pathogen *Cladosporium fulvum* is described. The interaction between the fungal pathogen *C. fulvum* and tomato is an excellent model system to study fungus - plant specificity. This will be demonstrated in Chapter 1, which gives an overview on the current status of the *C. fulvum* -tomato research.

Most of this thesis will deal with the avirulence gene *avr9* of *C. fulvum*. Chapter 2

describes the cloning of the cDNA encoding the AVR9 race-specific elicitor. Races of *C. fulvum* virulent on tomato genotypes carrying the complementary resistance gene *Cf9* lack the avirulence gene *avr9*. In Chapters 3 and 4 conclusive evidence is given that *avr9* is a genuine avirulence gene. Chapter 3 describes the transformation of races lacking the *avr9* gene with the cloned *avr9* gene, resulting in a change of cultivar-specificity from virulent to avirulent on tomato genotype *Cf9*. In addition, disruption of *avr9* in avirulent races of *C. fulvum*, as described in Chapter 4, resulted in disruption mutants which have now become virulent on tomato genotype *Cf9*.

The AVR9 race-specific peptide elicitor could be studied in more detail by high expression of the *avr9* gene in transformants of *C. fulvum*, as described in Chapter 5. Evidence is presented for the involvement of both the host plant tomato and the pathogen *C. fulvum* in the processing of the primary avirulence gene product to its mature AVR9 elicitor. The regulation of expression of the *avr9* gene is described in Chapter 6. Expression of *avr9* is induced *in planta* and under conditions of nitrogen limitation *in vitro* which might reflect the nutritional conditions which the fungus encounters in the tomato leaf.

In Chapter 7, the cloning and characterization of two putative pathogenicity genes of *C. fulvum* is described. These *ecp* (encoding extracellular proteins) genes are highly expressed *in planta*. Possible functions of the *ecp* genes are discussed and future research is described to elucidate their putative function in pathogenicity.

The results described in Chapters 1 to 7 are discussed in Chapter 8. The current physiological, biochemical and molecular knowledge on the *C. fulvum* - tomato interaction is presented in two models which describe basic compatibility and race-specific incompatibility, respectively.

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Chapter 1

The *Cladosporium fulvum* - tomato interaction, a model system for fungus - plant specificity

Guido F.J.M. van den Ackerveken and Pierre J.G.M. de Wit

adapted from *Pathogenesis and Host Specificity in Plant Diseases*
(Kohmoto, K., Singh, R.P., Singh, U.S. and Zeigler, R., eds.), Pergamon Press, Oxford, In press

Table of contents

I. Introduction

The fungal pathogen *Cladosporium fulvum* Cooke (syn. *Fulvia fulva*) is the causal agent of leaf mould of tomato (*Lycopersicon esculentum* Mill.). *C. fulvum* was first described by Cooke in 1883 [1], from diseased material collected in South Carolina (USA). The origin of *C. fulvum* is most probably South America, the centre of origin of tomato. The fungus is a biotrophic pathogen, which can however easily be grown in axenic culture. In infected tomato leaves, *C. fulvum* grows between the mesophyll cells and does not form specialized feeding structures such as haustoria.

Since the 1930s many genes for resistance to *C. fulvum* from related wild species of tomato have been transferred to cultivated tomato in breeding programmes [2]. Often within ten years after the introduction of these resistant cultivars, new races of *C. fulvum* appeared which could overcome the monogenic resistance [3,4]. At least eleven different genes for resistance have been described of which six are available in near-isogenic lines of tomato cultivar Moneymaker [5]. The observed differential interactions (compatible or incompatible) between different races of *C. fulvum* and near-isogenic lines of tomato suggested that the *C. fulvum* - tomato interaction fits the gene-for-gene hypothesis [6,7].

In the last two decades, the physiology, biochemistry and molecular biology of the *C. fulvum* - tomato interaction have been studied by several groups [8-10]. Fundamental questions on pathogenicity, specificity and plant defence have been actively pursued. In recent years we have gained more insight into basic compatibility and active plant defence, and have made significant progress in the molecular understanding of the gene-for-gene interaction and the role of fungal avirulence genes in pathogen recognition.

In this chapter we demonstrate that the interaction between *C. fulvum* and its host plant tomato is an excellent model system to study the gene-for-gene interaction. Pathogenicity and avirulence factors and their respective involvement in induction of susceptibility and resistance will be discussed in detail. We will only briefly discuss the defence responses triggered by *C. fulvum* as these responses are similar in other host-pathogen interactions which are studied worldwide by many groups (for reviews [11-13]). The host defence responses are in many cases not specific for one particular host-pathogen interaction, whereas their induction often is pathogen-specific in most plants. The induction of active plant defence, in particular the early responses and the hypersensitive response, by race-specific elicitors of *C. fulvum* will be discussed in more detail. The latest findings will be combined in a model explaining the interaction at the molecular and biochemical level.

II. Histopathological and ultrastructural aspects of pathogenesis

Cladosporium fulvum causes pale-yellow spots on the upper side of infected tomato leaves, which are the result of abundant fungal growth inside the leaf. After an incubation period of about two weeks conidiophores emerge through stomata and sporulation becomes visible as

brown-grey velvet areas at the lower side of the leaf. When the infection progresses, leaves become necrotic and eventually fall off.

The first detailed microscopic studies were performed by Bond in 1938 [14]. Germination, penetration, development of intercellular mycelium and sporulation were initially studied by light microscopy. Later, more detailed studies were performed by Lazarovits and Higgins [15,16] and De Wit [17] using transmission and scanning electron microscopy. The infection process was studied in time after inoculation of the lower side of leaves of near-isogenic lines of susceptible and resistant cultivars of tomato with conidiospores of virulent and avirulent races of *C. fulvum*. Conidiospores germinate on the leaf surface at high relative humidity and form slender germ tubes, the so-called runner hyphae (2-3 μm in diameter). These runner hyphae are not growing in the direction of the stomata, but rather continue to grow until they accidentally encounter open stomata, which are subsequently penetrated by the fungus at three to five days after inoculation (Fig. 1C). Once inside the leaf, the diameter of hyphae enlarges two to three fold (4-5 μm). The fungus colonizes the intercellular spaces in the leaves of a susceptible host without visible induction of defence responses (Fig. 1A). The hyphae are in close contact with mesophyll cells but do not form any specialized feeding structures such as haustoria (Fig. 1D). Extracellular proteins (ECPs, discussed in section IV B) produced by *C. fulvum* are associated with the matrix between fungal hyphae and host cell walls [18]. Whether these proteins are essential for the adherence of *C. fulvum* to the host cell wall is not known. Approximately 10 days after penetration, a network of hyphae appears in stomatal cavities and conidiophores emerge through the stomata and profusely produce conidiospores.

The fungus cannot reproduce on resistant cultivars of tomato. After penetration of the leaf through the stomata, fungal growth is arrested at an early stage (Fig. 1B). The extent of fungal growth is dependent on the avirulence gene - resistance gene combination, which probably determines the precise timing of induction of plant defence [19]. The defence response is characterized by the hypersensitive response (HR, observed as cell browning and necrosis), decompartimentalization and callose deposition in those host cells which are in close contact with the hyphae of avirulent races of the pathogen [15-17]. Mesophyll cells in or around the infection site often show abundant excretion of extracellular material which occurs more frequently in resistant than in susceptible plants [16].

Avirulent races of *C. fulvum* induce drastic changes in the leaves of resistant tomato cultivars, whereas virulent races do not induce any visible changes in the host under optimal conditions. In contrast, intercellular injection of conidia of both virulent and avirulent races of *C. fulvum* into leaf mesophyll induce localized responses such as cell wall appositions and browning [20]. A non-specific glycoprotein elicitor (discussed in section IV A) produced by the injected conidia of virulent as well as avirulent races is probably responsible for the initial induction of these responses [21-25]. Germ tubes of virulent races, however, appear to grow out of the responding leaf mesophyll zone and hyphae continue to colonize the tomato leaf without further induction of defence responses, whereas the growth of avirulent races is very quickly arrested.

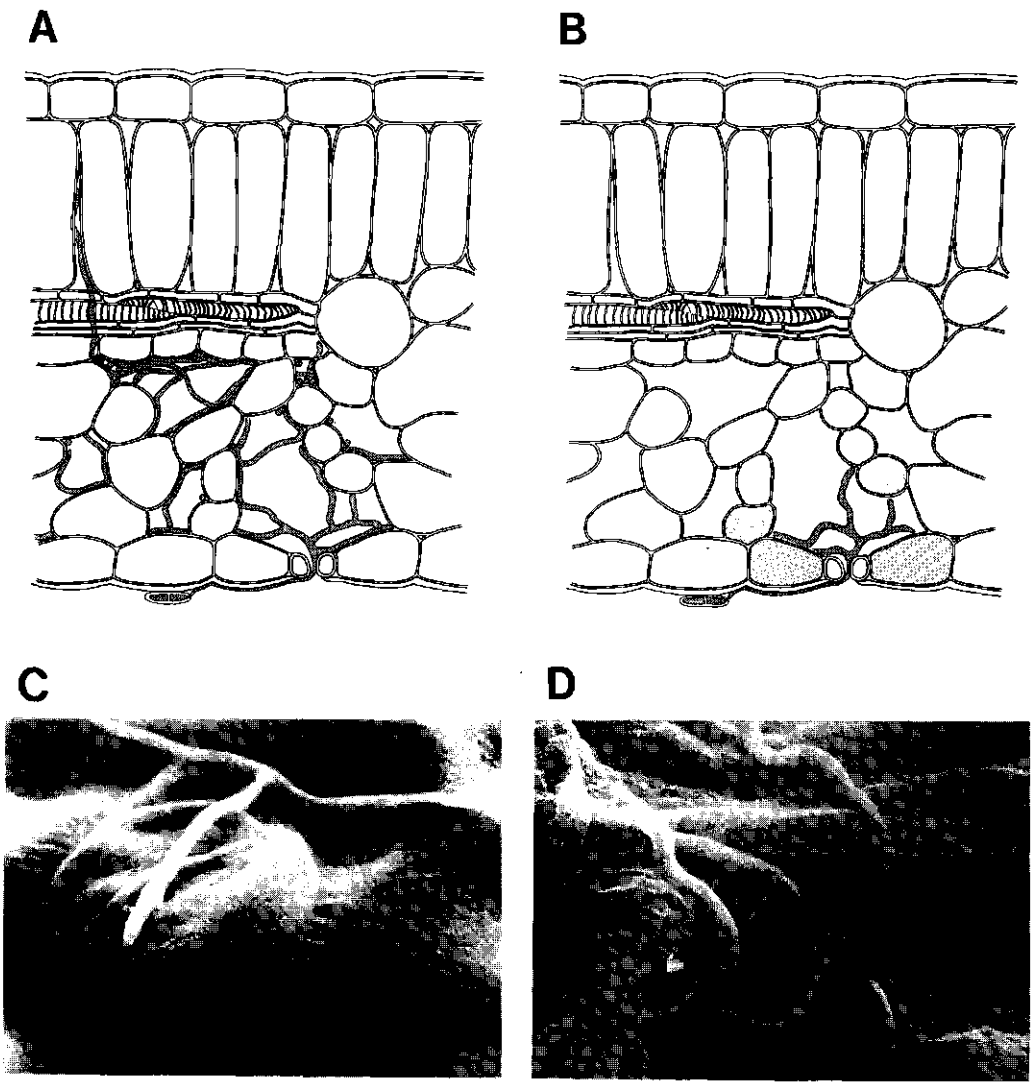

Figure 1. **A, B)** Schemetic representation of a transverse section of a tomato leaf 6-8 days after inoculation with *Cladosporium fulvum*. The fungal conidiospore germinates on the lower leaf surface, forms a thin runner hypha and enters the leaf through a stoma. The penetrating hypha develops into a thicker intercellular mycelium. **A)** Susceptible cultivar inoculated with a virulent race of *C. fulvum*. The fungus is growing abundantly in the intercellular spaces around the mesophyll cells; no visible defence responses are activated. **B)** resistant cultivar inoculated with an avirulent race of *C. fulvum*. After penetration fungal growth is arrested and mesophyll cells in contact with the fungus develop a hypersensitive response (HR, dark cells). **C)** Scanning electron micrograph showing penetration of *C. fulvum* through a stoma at the lower side of the tomato leaf. **D)** Scanning electron micrograph showing fungal hyphae growing around mesophyll cells (as in A).

Chitinases and 1,3-ß-glucanases of tomato, associated with plant defence [26,27], were studied by immunocytology using polyclonal antibodies [28]. Subcellular localization of proteins in the intercellular spaces of *C. fulvum*-infected tomato leaves is hindered by poor fixation of these often highly soluble proteins. No significant differential accumulation of chitinases and 1,3-ß-glucanases was observed between incompatible and compatible interactions. In addition, no association of chitinases and 1,3-ß-glucanases with fungal hyphae was observed, suggesting that these hydrolytic enzymes play a minor role in active defence of tomato against *C. fulvum*.

Future histopathological research on the *C. fulvum* - tomato interaction will involve molecular biological techniques. *In situ* mRNA hybridization using labeled RNA probes derived from plant defence genes (encoding e.g. chitinases or 1,3-ß-glucanases) will be used to study the temporal and spatial expression of these genes. An alternative approach is directed to use the reporter gene encoding the *Escherichia coli* ß-glucuronidase (*uid*A), which was demonstrated to be fully active in *C. fulvum* [29]. Fusion of promoters of interest to the *uid*A gene will allow to study the timing and location of expression of fungal genes involved in pathogenesis, and to search for compounds which induce those genes *in vitro*.

III. The genetics of the *Cladosporium fulvum* - tomato interaction

There has been a continuous selection and breeding of tomato for more than 200 years [2]. In the beginning of this century resistance to *C. fulvum* was first described and certain resistant varieties were selected. In the 1930s Langford began to breed for resistance and soon noticed that certain resistant varieties were still susceptible to some isolates of *C. fulvum* [30]. In the following decades many genes for resistance to *C. fulvum* (*Cf* genes) have been crossed into *Lycopersicon esculentum* from related wild species of *Lycopersicon* including *L. pimpinellifolium*, *L. hirsutum*, *L. hirsutum* var. *glabaratum* and *L. peruvianum* [2,31]. The *Cf* resistance genes are single dominant genes and have been mapped on different chromosomes of tomato [32,33]. Resistance genes *Cf2* and *Cf5* are allelic or closely linked and map on chromosome 6 of tomato [33]. Also, resistance genes against other pathogens, such as *Meloidogyne incognita* (*Mi*) [34] and *Oïdium lycopersicum* [35], map at around the same position on chromosome 6. The resistance genes *Cf4* and *Cf9* are also allelic or closely linked and map on chromosome 1 [32,36]. Near-isogenic lines of tomato cultivar MoneyMaker are available, each carrying one of these four *Cf* resistance genes, which are very useful for studying the mechanisms of resistance genes separately.

Disease resistance genes have not been isolated so far. Several groups are currently employing different strategies to clone *Cf* resistance genes [37]. Approaches used are (i) map based cloning, (ii) transposon tagging, (iii) functional cloning and (iv) cloning genes encoding receptors for race-specific elicitors (section IV D).

As described above, genes for resistance introduced in tomato were sooner or later

overcome by new races of *C. fulvum* [3,4]. The observed differential interactions (compatible or incompatible) between different races of *C. fulvum* and near-isogenic lines of tomato suggested that the gene-for-gene hypothesis was applicable to the *C. fulvum* - tomato interaction [6,7]. The gene-for-gene hypothesis states that genes for resistance in the plant match to genes for avirulence in the pathogen. Flor, in 1942 [38], developed the gene-for-gene hypothesis based on genetic studies of the *Melampsora lini* - flax interaction. Avirulence genes in *M. lini* and the corresponding resistance genes in flax where shown to be single loci, which segregated in a mendelian fashion [39].

The gene-for-gene hypothesis in the *C. fulvum* - tomato interaction is supported by the finding of race-specific elicitors (discussed in section IV C) which fit into the specific elicitor - specific receptor model [40,41]. In this model, the gene products of fungal avirulence genes, the so-called race-specific elicitors, are recognized by specific receptors in the resistant host leading to incompatibility (Fig. 2). The receptors recognizing the specific elicitors produced by the pathogen might be the direct products of resistance genes. Absence or mutation of either the avirulence gene in the pathogen or the corresponding resistance gene in the host will abolish recognition leading to compatibility. In this model the race-specific recognition is superimposed but fully dependent on basic compatibility.

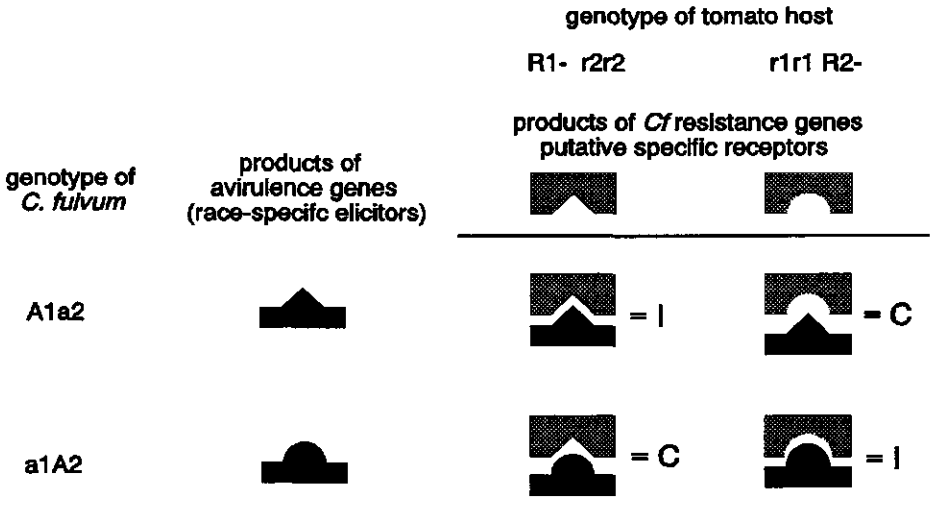

Figure 2. Schematic representation of the specific elicitor - specific receptor model explaining the gene-for-gene interaction between *Cladopsorium fulvum* and tomato. Avirulence genes (A1, A2) of the haploid fungal pathogen *C. fulvum* encode race-specific peptide elicitors which are recognized by the products of dominant *Cf* resistance genes (R1, R2) in tomato, the putative specific receptors. Recognition induces active plant defence (HR) resulting in incompatibility (I). Absence of the race-specific elicitor or the corresponding specific receptor will not result in recognition and *C. fulvum* continues to grow inside the tomato leaf without inducing any defence responses (compatibility, C).

The presence of single avirulence genes in *C. fulvum* cannot be demonstrated genetically, as the sexual stage of this haploid fungus is not known or non-existing. Day, in 1957, tried to prove the dominant character of an avirulence gene in *C. fulvum* [42]. Young sporulating cultures of a mutant strain containing a red pigment marker and avirulent on tomato variety Vetomold (carrying resistance gene *Cf2*) were irradiated with UV in order to obtain virulent mutants. One mutant was obtained which had become virulent on the previously resistant tomato variety Vetomold. This mutant still contained the red pigment marker, suggesting that it was a genuine mutant and not a contaminant. Mutation of *C. fulvum* from avirulence to virulence as described by Day [42], was the first indication for the existence of avirulence genes in *C. fulvum*.

Mutation to virulence as described above [42], is the most plausible explanation for the development of new races of *C. fulvum*. Alternatively, races with new specificities might arise as a result of parasexual recombination by hyphal anastomosis of two different races of *C. fulvum* [43]. However, it should be emphasized that only one report on occurrence of hyphal anastomosis in *C. fulvum* exists in the literature [43] which could not be repeated with well characterized mutants [10,44]. Therefore, the occurrence of parasexual recombination under natural conditions is very uncertain.

Another mechanism for the development of new races of *C. fulvum* might be transposition and integration of a transposable element in avirulence genes [45]. A retrotransposon-like element called *Cf*T-1 is present in 30-100 copies at a variety of sites in the genome of *C. fulvum* [45]. Evidence that *Cf*T-1 is a genuine LTR(long terminal repeat)-retrotransposon was obtained by demonstrating expression of *Cf*T-1 and packaging into virus-like particles [46]. However, no direct evidence for transposition of *Cf*T-1 was obtained, and only circumstantial evidence suggests that *Cf*T-1 has transposed recently [46]. The use of transposons to inactivate genes by disruption can be of great help for the detection and subsequent isolation of genes involved in pathogenicity and cultivar-specificity.

As mentioned above, no straightforward classic genetic studies can be performed with *C. fulvum*, since it is an imperfect fungus. An artificial method for genetic analysis of *C. fulvum* was performed by Talbot *et al.*[44]. By protoplast fusion of two parental mutants it was shown that a diploid strain could be obtained containing a mixed set of chromosomes from both parents. Since the diploid is not stable, haploidization occurred spontaneously. Recombinants were obtained from fusions between mutants in the nitrate assimilatory pathway. A genetic linkage map of *C. fulvum* is currently being constructed, using this forced parasexual recombination [47,48]. An inter-racial fusion between a mutant race 4 and a mutant race 5, containing avirulence gene *avr*5 and *avr*4 respectively, has been studied in detail [47,48]. Over 50 phenotypic, RFLP and RAPD markers have been used to analyse the haploid progeny. A linkage map containing seven linkage groups has been established. Unfortunately, no marker mapped to *avr*4 and *avr*5 [48].

An alternative strategy to isolate avirulence and pathogenicity genes might be map-based cloning by chromosome walking. However, this technique has not yet been successful for *C.*

fulvum for two major reasons. First, there is only a small number of polymorphic markers on the linkage map of *C. fulvum*, due to the low genetic variation among races of *C. fulvum*. Secondly, the high content of repetitive DNA present on all chromosomes of *C. fulvum* hampers chromosome walking [49a]. Hybridization of a genomic library of *C. fulvum* with labeled total genomic DNA revealed that 60 - 70 % of the clones contained repetitive DNA. The high degree of repeated sequences could also be a major reason for the problems encountered in employing genomic subtraction, which was initiated to get an overall impression of the occurrence of deletions in *C. fulvum* [50].

Eleven chromosomes of *C. fulvum* can be separated by pulsed-field gel electrophoresis [51]. The chromosomes range in size from about 2 to 7 Mbp (Fig. 3) and show few polymorphisms in the different races and isolates of *C. fulvum*. Some races contain an additional mini chromosome (nr. 12) of approximately 800 kb. All *C. fulvum* genes cloned so far have been mapped on different chromosomes and can be used as chromosome-specific markers. Additional chromosome-specific markers have been isolated from a genomic library of *C. fulvum* [49a], which can be used to identify individual chromosomes and to analyse chromosome length polymorphisms.

The well documented genetics of tomato and to some extent of *C. fulvum*, enables the study of resistance and avirulence. The availability of near-isogenic lines of tomato with different genes for resistance and of races of *C. fulvum* with different specificities makes the *C. fulvum* - tomato interaction an excellent model system to study gene-for-gene specificity.

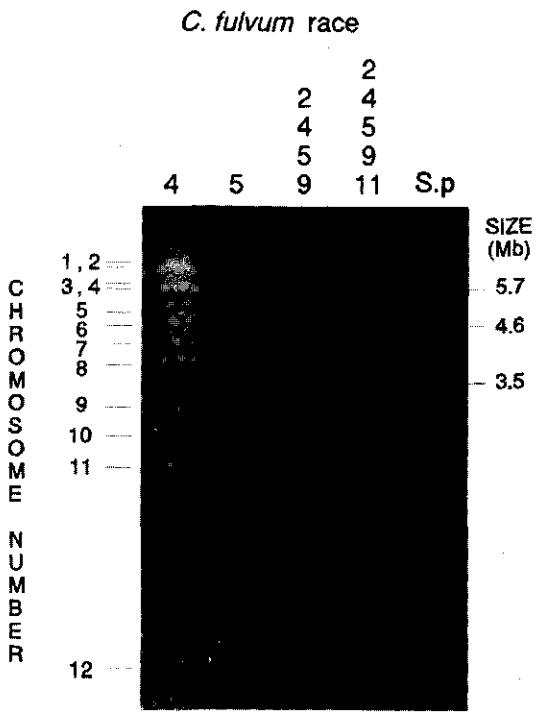

Figure 3. Separation of intact chromosomal DNA molecules of 4 different races of *Cladosporium fulvum* by pulsed-field gel electrophoresis . The size of the eleven chromosomes of *C. fulvum* are estimated to be 6.6, 6.3, 5.9, 5.7, 5.4, 4.6, 3.9, 3.4, 2.8, 2.5 and 1.9 Mb, respectively for chromosomes 1 to 11 [48]. An additional 'mini'-chromosome of 0.8 Mb is present in some races of *C.fulvum*. Chromosome numbering, based on the pattern obtained with race 4, is indicated on the left, the size of the *Schizosaccharomyces pombe* (Sp) chromosomes is indicated on the right.

IV. Biochemical and molecular aspects of the interaction

A. Cladosporium fulvum and tomato studied separately

The early studies on the *C. fulvum* - tomato interaction were focused on the individual partners. Resistant and susceptible plants were analysed chemically in search for factors which could influence the growth and development of *C. fulvum* [52,53]. The sugar and amino acid content of whole leaves of different tomato varieties were compared, but differences in chemical composition could not be correlated to resistance or susceptibility [52,53]. The supply of nutrients and light conditions during the growth of tomato plants, however, influence the severity of disease symptoms [54].

The early biochemical work on *C. fulvum* was mainly focused on factors released by the fungus in the culture fluid of *in vitro* grown mycelium. Glycoproteins produced by *C. fulvum in vitro* induced necrosis when injected in tomato leaves but were, however, not race-specific [21-25]. These non-specific elicitors induced physiological responses in tomato, such as phytoalexin accumulation [24], electrolyte leakage [55] and increased lipoxygenase and lipoperoxidase activity [55]. Interestingly, most of these responses were only observed in tomato and not in any other plant species tested [22]. The activity of the non-specific glycoprotein elicitors could be suppressed by incubating them with intercellular fluid collected from *C. fulvum*-infected tomato leaves [56] . A host-derived factor was demonstrated to inactivate the non-specific glycoprotein elicitor by degradation. Also, a non-proteinaceous low molecular weight suppressor of the non-specific glycoprotein elicitor was characterized recently [57]. Since the products of digestion of polypectate had similar effects it was suggested that this suppresssor originates from action of pectolytic enzymes on host cell walls. The presence of this suppressor in the apoplast of healthy tomato leaves, together with the host-produced elicitor degrading activity, suggest that under natural conditions the non-specific glycoprotein elicitor does not play a major role in the *C. fulvum* - tomato interaction.

Transformation of *C. fulvum* has been established using protoplasts [58,59]. Dominant selection markers, such as the hygromycin resistance marker (*hph*) [60] and the phleomycin resistance marker (*phleo*R) [61] have been successfully used. High transformation frequencies were obtained by using a complementing gene as a selection marker for transformation of auxotrophic mutants of *C. fulvum*. Uridine auxotrophic mutants of *C. fulvum*, resistant to fluoro-orotic-acid, were complemented to prototrophy by transformation with heterologous *pyr* genes of *Aspergillus nidulans*, *A. niger* and *Claviceps purpurea* [49,62]. The isolation of the *pyr* gene of *C. fulvum* will facilitate the development of an efficient gene targeting system [63], which can be used in future research to study the promoters of avirulence and pathogenicity genes of *C. fulvum*.

B. Basic compatibility

The ability of a pathogen to successfully penetrate, colonize and reproduce on a certain host plant is called basic compatibility [64-66]. Only a few fungi are pathogens and are able to establish and maintain basic compatibility on a certain host species. In addition, biotrophic plant pathogens are fully dependent on the living host and are therefore often highly specialized. *C. fulvum* is a good example of a very specialized biotrophic pathogen which can only colonize tomato. Until now, tools employed by *C. fulvum* to successfully infect tomato, the so-called pathogenicity factors, are not known. *C. fulvum* grows exclusively in the intercellular spaces of the tomato leaf without causing any visible damage to host cells. Factors involved in pathogenicity are therefore likely to be produced in the intercellular spaces of the tomato leaf, the interface between pathogen and host. Intercellular fluid (IF) containing these factors can be isolated by infiltrating leaves of compatible tomato - *C. fulvum* interactions with water or buffer *in vacuo*, followed by a centrifugation step [67]. Obviously, IF contains besides fungal compounds also plant compounds.

From this IF, several low molecular weight proteins (<20 kD) have been purified, which are most probably of fungal origin since they are not present in the incompatible *C. fulvum* - tomato interactions [68]. These extracellular proteins (ECPs) are also not produced by *C. fulvum* grown *in vitro*. Two of these proteins, ECP1 (formerly called P1)[69] and ECP2 [18] were purified and the corresponding genes, *ecp*1 and *ecp*2, were subsequently isolated [49,70]. Analysis of DNA sequences and derived amino acid sequences of the two *ecp* genes have not given any clue to their possible enzymatic or structural functions. In all races of *C. fulvum* the *ecp* genes are highly expressed *in planta* as compared to *in vitro* (Fig. 4). This high expression together with the abundance of the ECPs in the extracellular space of infected tomato leaves suggest a role in pathogenicity. The extracellular localization could indicate a role in the matrix which is present at the interface between the fungal hyphae and the host cell wall [18]. Possibly, the ECPs are actively interfering with the metabolism and/or transport of host nutrients within the tomato leaf, or suppressing non-specific plant defence responses. Disruption of the *ecp* genes by gene replacement with mutated *ecp* genes will reveal whether these genes are essential for pathogenicity of *C. fulvum*.

Several genes of *C. fulvum* have been isolated (*avr*4 [71], *avr*9 [49,72], *ecp*1 and *ecp*2 [49,70]) which are highly expressed *in planta*. Figure 4 shows the expression of these four genes in *C. fulvum* grown *in vitro*, an incompatible and a compatible *C. fulvum* - tomato interaction. The *ecp* and *avr* genes are highly expressed during growth *in planta* but hardly or not during growth *in vitro*. Of the four genes, only avirulence gene *avr*9 shows induced expression *in vitro* under conditions of nitrogen starvation [49]. The mechanisms of induction or derepression are not known. The promoters of the four genes do not reveal common structural motifs, which could be involved in the regulation *in planta*, although small homologies are present between *ecp*1 and *ecp*2 [49,70]. Future research using promoter-reporter gene fusions will be used to define the promoter regions which are involved in the regulation of transcription.

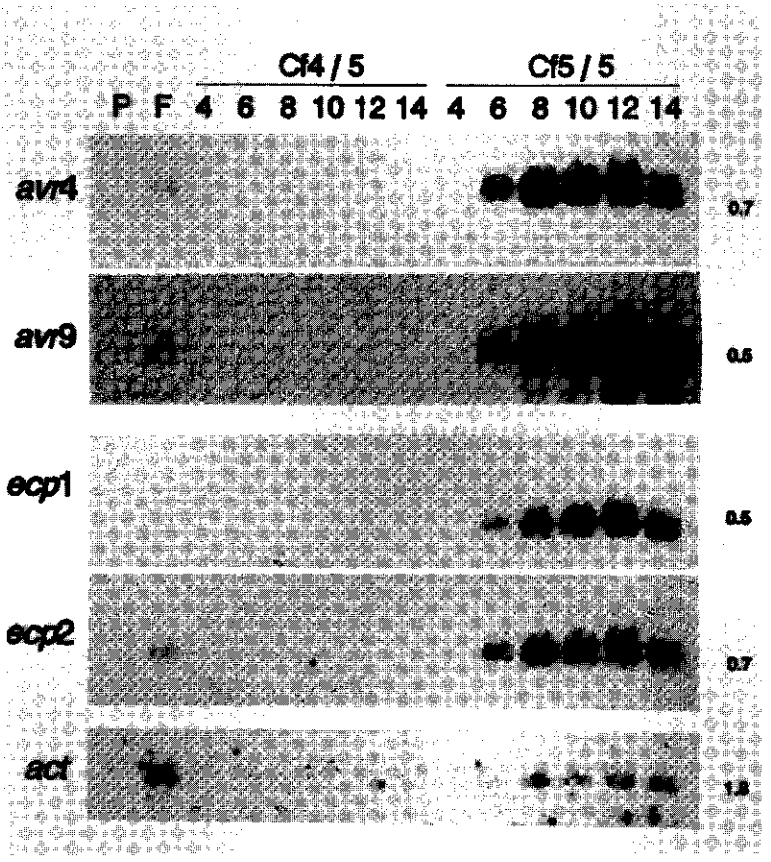

Figure 4. Northern blot analysis of poly(A)$^+$RNA isolated from uninoculated tomato genotype *Cf5* (lane P), from *Cladosporium fulvum* grown *in vitro* (lane F), from an incompatible interaction tomato *Cf4/ C. fulvum* race 5 (Cf4/5) and a compatible interaction tomato *Cf5/ C. fulvum* race 5 (Cf5/5) at different times after inoculation (4, 6, 8, 10, 12 and 14 days, respectively). The blot was hybridized with [α-^{32}P]dATP-labeled DNA probes of the *C. fulvum* genes *avr*4, *avr*9, *ecp*1, *ecp*2 and *act* (actin gene [49a]). The latter was used as a constitutive control for fungal mRNA [70].

Since *C. fulvum* obtains its nutrients from the apoplast, Joosten *et al.*[73] determined the mono- and di-saccharides present in the intercellular fluid (IF) of *C. fulvum*-infected tomato leaves compared to those present in IF of healthy leaves. IF of tomato leaves inoculated with an avirulent race of *C. fulvum* had a carbohydrate composition similar to IF of healthy tomato leaves and remained unaffected within 14 days post inoculation. The carbohydrate composition of IF isolated from tomato leaves heavily colonized by *C. fulvum* was completely different. In a time course experiment, a twofold increase in sucrose during the first seven days was followed by a decrease. The level of fructose and glucose increased tenfold between

five and nine days after inoculation, followed by a decrease. Mannitol, which is absent in healthy tomato leaves, accumulates strongly until nine days after inoculation, to reach a maximum at 14 days after inoculation. These results suggest that early in the interaction invertases of *C. fulvum* hydrolyze sucrose into fructose and glucose. The fungal enzyme mannitol dehydrogenase is subsequently involved in the formation of mannitol, which probably can only be utilized by the fungus but not by the host plant.

C. *Race-specific incompatibility*

A major breakthrough in the research on the *C. fulvum* - tomato interaction was the discovery of race-specific elicitors, the inducers of active plant defence, in IF of *C. fulvum*-infected tomato leaves [67]. Injection of IF in the leaves of resistant tomato plants resulted in the induction of HR, which is visible as chlorosis or necrosis. Proteinaceous compounds inducing a HR on resistant tomato cultivars were clearly demonstrated to be correlated with the presence of avirulence genes in certain races of *C. fulvum* [74,75]. One such race-specific peptide elicitor, the product of the avirulence gene *avr9*, was purified from IF of *C. fulvum*-infected tomato leaves [76]. The purified peptide elicitor specifically induces HR (observed as extensive necrosis) on tomato plants carrying the complementary resistance gene *Cf9*. Races virulent on tomato genotype *Cf9* do not produce the elicitor [77]. Recently, the race-specific elicitor inducing HR on tomato genotype *Cf4*, the product of *avr4* has been purified [71].

The race-specific elicitor inducing HR on tomato genotypes containing the resistance gene *Cf9* was purified and the amino acid sequence was determined [78]. The corresponding cDNA was isolated using oligonucleotide probes deduced from the amino acid sequence of the peptide [49,72]. The gene encoding the elicitor, named *avr9*, was only detected in fungal races which are avirulent on tomato genotypes carrying the *Cf9* gene. Races virulent on *Cf9* genotypes of tomato have evaded recognition by the host as they lack the entire *avr9* gene [49,72] and therefore do not produce the peptide elicitor. The *avr9* gene was subsequently isolated from a genomic library of *C. fulvum* and sequenced [49,79]. To demonstrate the causal relationship between a functional *avr9* gene encoding the elicitor and avirulence on tomato genotype *Cf9*, the cloned *avr9* gene was transferred to race 2.4.5.9.11 of *C. fulvum*, which lacks the *avr9* gene and can overcome the resistance genes *Cf2*, *Cf4*, *Cf5*, *Cf9* and *Cf11* of tomato. The cultivar-specificity of the transformants containing the *avr9* gene changed from virulent to avirulent on tomato genotype *Cf9*. This was the first report on the cloning of a fungal avirulence gene, which demonstrated that a single gene is responsible for changes in race-cultivar specificity [49,79].

Disruption of *avr9* in two races of *C. fulvum* was achieved by replacing the *avr9* coding region by the *Aspergillus nidulans pyrG* gene via a gene-replacement strategy in a pyr$^-$ mutant [49,62]. The cultivar-specificity of these disruptants was consequently changed from avirulent to virulent on tomato genotype *Cf9*, confirming the conclusions drawn from the transformation experiments described above [49,79].

The elicitor encoded by *avr*9 can be isolated from IF of *C. fulvum*-infected tomato leaves [78], but not from *C. fulvum* grown *in vitro*. To circumvent the laborious isolation procedure of the AVR9 elicitor from infected plants and to obtain large quantities of this elicitor, we have constructed a fusion between a strong constitutive promoter (*gpd* from *Aspergillus nidulans* [80]) and the *avr*9 coding region [49,79]. *C. fulvum* transformed with this *avr*9-construct produces large amounts of active AVR9 elicitor *in vitro* [49,81]. Milligrams of the AVR9 peptide have been purified from culture filtrates of these transformants, which can be used for AVR9 structure analysis (NMR) and elicitor-receptor binding studies. Plant breeders on the other hand use this AVR9 elicitor in breeding programmes to screen their entries for the presence of the *Cf9* resistance gene.

Recently, the avirulence gene *avr*4 of *C. fulvum* has been cloned by a similar approach [71]. Preliminary results indicate that recognition by tomato genotypes carrying the resistance gene *Cf4* is overcome by single base pair mutations in the coding sequence of the gene. In all virulent races analysed, these mutated genes contain one point mutation, but the mutations can be at four different locations in the protein. All mutations, however, involve one change from cysteine to tyrosine. In one case the change from avirulence to virulence was caused by a frame shift mutation.

D. Recognition and defence responses

Biochemical research on the mechanism(s) of resistance to *C. fulvum* was focused on the differences between incompatible (avirulent race, resistant cultivar) and compatible (virulent race, susceptible cultivar) *C. fulvum* - tomato interactions. The differential accumulation of phytoalexins [82,83] and pathogenesis related (PR) proteins, such as P14-isomers [84] and 1,3-ß-glucanases and chitinases [26,85], was clearly demonstrated. The general idea is that in the incompatible interaction an avirulent race is recognized at an early stage by the resistant plant leading to the induction of active defence responses.

The primary recognition of race-specific elicitors and the subsequent signal transduction is studied by several groups at the moment [19,86]. Tomato suspension cells treated with IF containing race-specific elicitors of *C. fulvum* were shown to have retained the specificity of the intact plants [86]. In contrast to the HR response of intact plants, the suspension cells retained their viability after treatment with IF. Vera-Estrella *et al.* [86], demonstrated that IF containing the AVR5 elicitor induced several physiological defence related responses, specifically in cell suspension cultures of tomato containing the *Cf5* resistance gene but not in cultures containing the *Cf4* resistance gene. Active oxygen species are rapidly generated after treatment of suspension cells with IF, and are associated with lipid peroxidation, cytochrome *c* reduction, luminol-dependent chemiluminescence, oxygen uptake and release of extracellular peroxidase and phenolic compounds. Extracellular production of active oxygen species seems to be an important factor in the initial reaction of tomato cells to specific elicitors of *C. fulvum*. The tomato cell suspension cultures responded in a similar

way to non-specific glycoprotein elicitors (section IV A) except for the luminol-dependent chemiluminescence and increase, rather than decrease, in oxygen uptake [87].

Using an *in vivo* cotyledon assay, Hammond-Kosack *et al.* [19], confirmed the formation of active oxygen species (refered to as oxidative burst) after injection of IF, containing AVR9 elicitor, in cotyledons of tomato seedlings carrying the *Cf9* resistance gene. This oxidative burst was detected within three hours after injection of elicitor. The production of ethylene was observed at nine to ten hours after injection, followed by increasing levels of salicylic acid after 12 hours. By 20 to 24 hours most mesophyll cells had lost membrane integrity, leading to electrolyte leakage and alkalinization. Macroscopically visible necrosis could be observed circa 30 hours after injection with IF.

The early responses described above are induced rapidly after treatment with race-specific elicitors. The mechanism of primary recognition of elicitors by the resistant plant, however, is still not known. Our laboratory is currently employing radioactively labeled AVR9 race-specific elicitor as a ligand to study the binding to potential receptors in tomato.

The knowledge on avirulence genes and race-specific elicitors of the pathogen has resulted in a strategy to develop disease-resistant crop plants. In the so-called two-component sensor system [37], the avirulence gene in combination with the corresponding resistance gene is used to convert non-specific pathogen recognition into rapid and effective plant defence. The gene encoding the AVR9 race-specific elicitor fused to a pathogen-inducible promoter will be expressed immediately after pathogen attack in transgenic plants. The AVR9 peptide will then be quickly recognized, leading to the inducion of active plant defence (HR) which will restrict the pathogen to the infection site. So far, transgenic tobacco plants containing *avr9* producing biologically active AVR9 elicitor are available [88]. Hopefully, in the near future transgenic tomato plants will be developed containing this two-component sensor system which will be resistant to various pathogens.

V. Concluding remarks

The data presented in this chapter clearly show that the *C. fulvum* - tomato interaction is a very useful model system to study fungus - plant specificity. Further unravelment of this model system may help us to answer basic questions on mechanisms of specificity and basic compatibility, not only for the *C. fulvum* - tomato interaction, but also for other fungus - plant interactions.

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Chapter 2

Cloning and characterization of the cDNA encoding the AVR9 race-specific elicitor of *Cladosporium fulvum*

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Summary

A race-specific peptide elicitor from *Cladosporium fulvum* induces a hypersensitive response on tomato genotype *Cf9* . We have hypothesized that the avirulence of fungal races on genotype *Cf9* is due to the production of this elicitor by an avirulence gene, *avr9*. In order 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 a mRNA of 500 nt. 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 C-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 which is absent in fungal races which are virulent on tomato genotype *Cf9*. Possible functions of the avirulence gene are discussed.

Introduction

Specialization of plant pathogens has resulted in the evolution of various formae speciales (fungi) and pathovars (bacteria) which are able to colonize only one or a small number of host plants. Within one forma specialis or pathovar, races can be found which 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 race-cultivar specific plant-pathogen interactions can be described by a gene-for-gene model, where a pathogen-encoded avirulence gene product interacting with the corresponding plant-encoded resistance gene product 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 (Hitchin *et al.*, 1989; Shintaku *et al.*, 1989; Staskawicz *et al.*, 1984, 1987; Vivian *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 carry out 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* (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 in order to study genes involved in pathogenicity and avirulence.

Our work on the tomato pathogen *Cladosporium fulvum* (syn. *Fulvia fulva*) has resulted in the identification of fungal race-specific elicitors which 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 which are avirulent on tomato genotype *Cf9*, but not in any interaction involving fungal races which are virulent on tomato genotype *Cf9* (Scholtens-Toma *et al.*, 1989). In this

paper, we report the cloning and characterization of the cDNA from the avirulence gene *avr*9, which is the first fungal avirulence gene to be cloned.

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 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 a mRNA of approximately 500 nucleotides (nt.) present in two compatible interactions: tomato genotype *Cf4* / *C. fulvum* race 4 (lane 3) and tomato genotype *Cf5* / *C. fulvum* race 5 (lane 4). This mRNA was not detected in uninoculated tomato genotype *Cf5* (lane 1) nor in *C. fulvum* grown *in vitro* (lane 2). Also, no hybridization was observed in the interaction tomato genotype *Cf5* / *C. fulvum* race 2.4.5.9.11 (lane 5), as would be expected for an interaction involving a race which is virulent on tomato genotype *Cf9*. 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 Fig. 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 tomato genotype *Cf5* with either *C. fulvum* 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 PAGE gel. Figure 3 shows that a specific extension product was synthesized on poly(A)$^+$RNA from the interaction tomato genotype *Cf5* / *C. fulvum* race 5 (lane 1), but not on poly(A)$^+$RNA from the interaction tomato genotype *Cf5* / *C. fulvum* race 2.4.5.9.11 (lane 2). The length of the extension product is approximately 270 nt., indicating that the *avr*9 mRNA contains approximately 200 nt. upstream of the sequence encoding the necrosis-inducing peptide.

Preparation and initial screening of cDNA library

Poly(A)$^+$RNA from the interaction tomato genotype *Cf5* / *C. fulvum* race 5 (represented in Fig. 2, lane 4) was used to prepare a cDNA library in λgt11. A library was obtained containing 100,000 independent recombinants. Screening of filters containing 5,000 phages with 5'-end-labeled oligonucleotide probe B resulted in the isolation of two possible candidates, one hybridizing weakly (phage A9-1), the other hybridizing significantly better

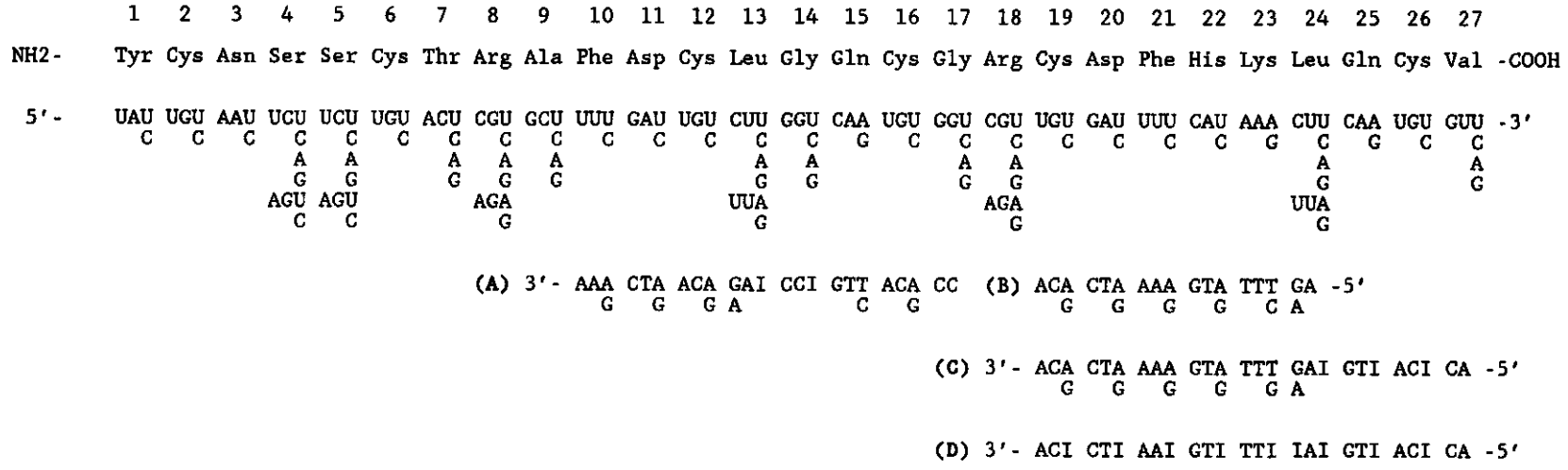

Figure 1. Amino acid sequence of the race-specific AVR9 elicitor with the corresponding coding sequences. The four oligonucleotide probes (A-D) used in this study are shown below. Degeneration of the probes at ambiguous positions was obtained by incorporating mixtures of dNTPs as indicated. In probes A, C and D, inosine (I) was incorporated in some positions.

Figure 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 genotype *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).

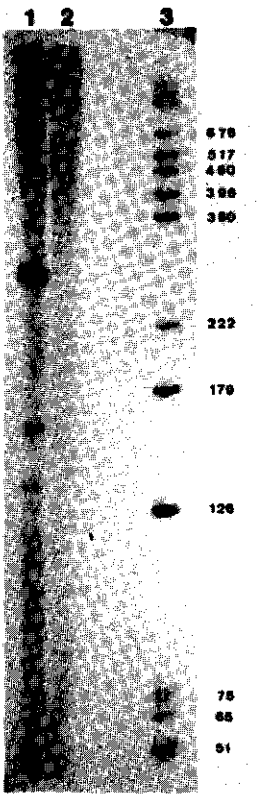

Figure 3. Primer extension with oligonucleotide probe B. Probe B was 5'-end labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase, hybridized to 5 μg of poly(A)$^+$RNA and extended with avian myeloblastosis virus reverse transcriptase. The extension products were analyzed on a denaturing 6% polyacrylamide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

(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 Fig. 2. Phage A9-1 hybridized with a mRNA of low abundance of 1.5 kb present in all three tomato - *C. fulvum* interactions (data 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 a mRNA of approximately 600 nt. present only in the compatible interactions tomato genotype *Cf4* / *C. fulvum* race 4 and tomato genotype *Cf5* / *C. fulvum* race 5, i.e. 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 cloning. The insert was estimated to be 400 bp.

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 bp. long and corresponds to the 3' end of the mRNA, including a poly(A)-tail of 20 nt. 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 nt. of the 5'-end of the mRNA. In order to obtain full-length cDNA clones, the cDNA library was screened again with a labeled RNA probe containing 70 nt. 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

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  1  XXXXXXXCGAAUUUCGAAAAAUCCUCAAGCCUACUAAGGUCUUUGUAGCCAUAGUCAUUU

 61  UAAUAAGUCUAUCGACUUACGUAUCUAUCAUGAAGCUUUCCCUCCUUAGCGUAGAGCUUG
                                  M  K  L  S  L  L  S  V  E  L  A

121  CUCUCCUAAUUGCUACUACUCUCCCACUUUGCUGGGCAGCUGCCCUCCCUGUAGGAUUGG
       L  L  I  A  T  T  L  P  L  C  W  A  A  A  L  P  V  G  L  G

181  GAGUCGGGCUAGACUACUGUAACUCAAGUUGUACUAGGGCCUUCGACUGUUUGGGUCAAU
       V  G  L  D  Y  C  N  S  S  C  T  R  A  F  D  C  L  G  Q  C

241  GUGGCAGAUGCGACUUUCAUAAGCUACAAUGUGUCCACUAGAGGACUACAGAGGAAGUGG
       G  R  C  D  F  H  K  L  Q  C  V  H

301  AGAGAAGAGGAGGGGAGAGGUACGAUAACUAGCGAGUAAAUCGUACAGGUAGAAAGGGAU

361  AGUAAGCAGGCAGAUAGACGGACGACGUUGCGACCUUAUCCAACAUAAGUCCUAGUCGUA

421  ACAUUCGUUCAUAUUGAAGGCUUUUCCUCAAUAGUUUCUCAAAUGUGCUGCGAGGCGCAG

481  AGCCAAG  487
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Figure 4. Nucleotide sequence of the *avr9* mRNA and the amino acid sequence of the *avr9* translation product (GenBank/EMBL accession number M55289). In the amino acid sequence, the mature necrosis-inducing peptide is indicated in bold characters.

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 Fig. 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 7 nt. of the *avr9* mRNA could not be read. Three minor extension products were observed which were 5-20 nt. 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 size (results not shown). The nucleotide sequence of the *avr9* mRNA as obtained from sequencing cDNA clones and mRNA is shown in Figure 4.

Timing of expression of the avr9 mRNA

To determine the timing of expression of the *avr9* gene, 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 tomato genotype *Cf5* / *C. fulvum* race 5, and another one of the incompatible interaction tomato genotype *Cf4* / *C. fulvum* race 5. Poly(A)$^+$RNAs from uninoculated tomato (tomato genotype *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 post inoculation, strongly increasing from day 8 post inoculation and onward. No hybridization could be observed in the incompatible interaction. A low amount of mRNA could be detected in the fungus grown *in vitro* (lane F).

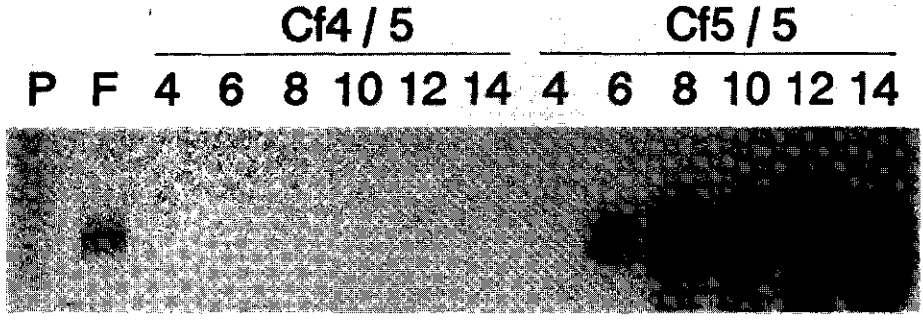

Figure 5. Time course of accumulation of the *avr9* mRNA. The insert of clone A9-2 was labeled and hybridized to a northern blot containing equal amounts of poly(A)$^+$RNA isolated from uninoculated tomato genotype *Cf5* (lane P), *Cladosporium fulvum* grown *in vitro* (lane F), an incompatible interaction between tomato genotype *Cf4* and *C. fulvum* race 5 (lanes Cf4/5) or from a compatible interaction between tomato genotype *Cf5* and *C. fulvum* race 5 (lanes Cf5/5) at different times (4, 6, 8, 10, 12 and 14 days, respectively) after inoculation as indicated.

However, when comparing mRNA levels of the fungus grown *in vitro* to the fungus grown *in planta*, it should be considered that the RNA samples from the compatible interaction contain only low 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 which 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 Fig. 6 (results not shown). Apparently, the coding sequence of the *avr9* gene is present in races that are avirulent on tomato genotype *Cf9* and absent in races that are virulent on tomato genotype *Cf9*.

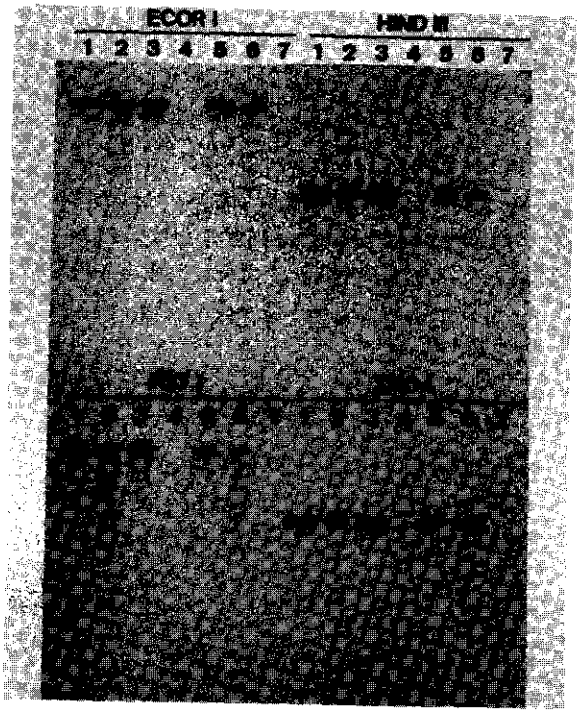

Figure 6. Southern blot analysis of total DNA from different races of *Cladosporium fulvum*. Races used were race 2 (lane 1), race 4 (lane 2), race 5 (lane 3), race 2.5.9 (lane 4), race 2.4 (lane 5), race 2.4.5 (lane 6) and race 2.4.9.11 (lane 7). Five micrograms of fungal DNA was digested with *Eco*RI, *Hin*dIII, *Pst*I or *Xho*I as indicated, separated on a 0.7% agarose gel and blotted onto a Hybond N^+ membrane. The blot was hybridized with a random-primed labeled fragment of 420 nucleotides containing the entire *avr9* cDNA insert (from A9-2).

Discussion

Using an oligonucleotide probe, we have detected the mRNA and cloned the cDNA encoding the necrosis-inducing peptide, which we hypothesize is the direct product of the avirulence gene *avr9* of *Cladosporium fulvum*. We believe that this gene is the avirulence gene since its gene product specifically induces HR on tomato genotype *Cf9*. Nevertheless, it remains to be proven that transformation of *C. fulvum* races, lacking *avr*9, with the *avr*9 gene confers avirulence on tomato genotype *Cf9* 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 C-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 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 *avr*9 elicitor is 3192 Daltons. The presence of the extra histidine probably explains why a synthetic peptide of 27 amino acids (lacking the C-terminal histidine) was not biologically active in bio-assays on tomato genotype *Cf9* (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 27 amino acid synthetic peptide by reduction and slow oxidation, but we never obtained a preparation which 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). The biological activity of the chemically synthesized peptide of 28 amino acids will be tested in future experiments. The N-terminal amino acids which are absent in the mature elicitor have some characteristics of a signal peptide (length and hydrophobic character). The cleavage site (between an aspartate 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 *avr*9 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 post inoculation and onwards. This increase might reflect the fast increase in fungal biomass during this period, as deduced from two parameters for fungal biomass *in planta*, mannitol concentration and mannitol dehydrogenase (MTLDH) activity in apoplastic fluids of infected leaves. These two parameters show a fast increase between day 6 and day 8 post inoculation (Joosten *et al.*, 1990). Before day 6 post inoculation, no significant fungal biomass could be measured in compatible interactions and consequently no *avr*9 mRNA could be detected. Also in the incompatible interaction, no fungal biomass and no *avr*9 mRNA could be detected (Fig. 5). Nevertheless, it is very likely that the *avr*9 gene

is expressed at very early stages of the infection cycle, since its gene product is believed to trigger HR on tomato genotype *Cf9* at about day 4 post inoculation (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 fungal point of view 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 not essential or can be compensated by other proteins. Whatever may be its function, the mode of action of the *C. fulvum avr9* gene product differs in several aspects from bacterial avirulence gene products. First, the mature *avr9* protein itself directly induces HR on tomato genotype *Cf9* (Scholtens-Toma and De Wit, 1988). Purified proteins encoded by bacterial avirulence genes do not induce 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). Secondly, the *avr9* gene product is a protein which 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 which do not produce the *avr9* elicitor, and are consequently virulent on tomato genotype *Cf9*, 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 non-functional allele which 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* (Staskawicz *et al.*, 1984; Staskawicz *et al.*, 1987). In contrast, non-functional alleles of avirulence genes were demonstrated in the plant pathogenic bacteria *Xanthomonas campestris* pv. *vesicatoria* (Kearney *et al.*, 1988) and *Pseudomonas syringae* pv. *glycinea* (Kobayashi *et al.* 1989; Kobayashi *et al.*, 1990b). In spontaneous mutants of *Xanthomonas campestris* 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 *Pseudomonas syringae* pv. *tomato*, other pathovars of *P. syringae* (e.g. pv. *glycinea*) appeared to have a homologous allele (Kobayashi *et al.*, 1989; Kobayashi *et al.*, 1990b) which seems 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 carried out. It remains to be determined whether virulence of other, if not all, fungal 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 AVR9, 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, in order to 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* (Van den Ackerveken *et al.*, this thesis). The structure and regulation of expression of the *avr9* gene will be an object of further studies.

Experimental procedures

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

Race	Avirulence genotypes	Interaction with tomato genotype *Cf9*
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[b]
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;
[b]C = compatible interaction, fungal race virulent.

Plants, fungi and inoculations

Near-isogenic tomato cultivars were grown as described by De Wit and Flach (1979). Different races of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* (Cooke) Cif) 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 tomato genotype *Cf9* are shown in Table 1.

RNA isolation and northern blotting

RNA was isolated by homogenizing tissue in guanidine-HCl buffer (8M guanidine-HCl/ 20 mM Mes/ 20 mM EDTA/ 50 mM ß-mercaptoethanol, pH 7), extracting with phenol/chloroform (1:1) and chloroform, and precipitating overnight with 2M 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 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 6xSSC/0.5% SDS/0.1% $NaPP_i$/100 μgml^{-1} calf thymus DNA/25 μgml^{-1} yeast tRNA at 35°C. The blots were washed in 1xSSC/0.5% SDS at 35°C. Hybridization of filters containing cDNA recombinant phages was performed in 6xSSC/0.5% SDS/0.1% $NaPP_i$/100 μgml^{-1} calf thymus DNA at 32°C. The filters were washed under the same conditions. 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). Hybridization was carried out at 42°C in 5xSSC/0.5% SDS/5xDenhardts/100 μg ml^{-1} calf thymus DNA. Filters were washed at 60°C in 0.2xSSC/0.2% SDS (high stringency) or at 50°C in 1xSSC/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 ng of labeled primer were hybridized to 5 μg of poly(A)$^+$RNA in 2x Reverse Transcriptase buffer (Promega) by heating to 65°C and cooling down to room temperature. The primer was extended with AMV Reverse Transcriptase (Promega) either in the absence or in the presence of dideoxynucleotides for RNA sequencing. The concentrations of ddNTP's 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 tomato genotype *Cf5* / *C. fulvum* race 5 using 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) and cDNA was ligated to *Eco*RI-digested lambda gt11 arms (Promega). After

ligation phage DNA was packaged in phage particles using a Packagene kit (Promega) and plated on *E. 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 carried out 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 *Cladosporium 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 either *Eco*RI, *Hin*dIII, *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 10xSSC onto Hybond N^+ membranes (Amersham) using a vacuum blotter (Millipore).

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Chapter 3

Molecular analysis of the avirulence gene *avr9* of *Cladosporium fulvum* fully supports the gene-for-gene hypothesis

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adapted from *The Plant Journal 2 (1992), 359-366*

Summary

The interaction between the fungal pathogen *Cladosporium fulvum* and tomato is supposed to have a gene-for-gene basis. Races of *C. fulvum* which have 'overcome' the resistance gene *Cf9* of tomato, lack the avirulence gene *avr9* which encodes a race-specific peptide elicitor. Races avirulent on tomato genotypes carrying the resistance gene *Cf9* produce the race-specific peptide elicitor, which induces the hypersensitive response (HR) on those genotypes. The causal relationship between the presence of a functional *avr9* gene and avirulence on tomato genotype *Cf9* was demonstrated by cloning of the *avr9* gene and subsequent transformation of *C. fulvum*. A race virulent on tomato genotype *Cf9* was shown to become avirulent by transformation with the cloned *avr9* gene. These results clearly demonstrate that the *avr9* gene is responsible for cultivar-specificity on tomato genotype *Cf9* and fully support the gene-for-gene hypothesis. The *avr9* gene is the first fungal avirulence gene to be cloned.

Introduction

The gene-for-gene hypothesis, which was proposed more than fifty years ago, states that for each gene conditioning race-specifc resistance in the host plant, there is a corresponding gene conditioning avirulence in a race of the pathogen (Flor, 1942). Gene-for-gene complementarity occurs most frequently in plant-pathogen interactions involving obligate and biotrophic pathogens which are highly specialized and have a narrow host range (Ellingboe, 1976; Heath, 1981; Keen, 1982). Resistance in host plants against these pathogens is usually inherited as a monogenic trait and is based on early recognition of the pathogen leading to a quickly induced hypersensitive response (HR) which restricts the pathogen to the infection site by local necrosis of a few host cells (De Wit, 1986; Keen, 1982; Keen, 1990; Pryor, 1987). The prevailing model in which recognition in gene-for-gene systems has been described is the elicitor-receptor model which states that a specific receptor in the resistant host plant interacts with a molecule of the pathogen, the so-called race-specific elicitor, leading to the induction of HR accompanied by a cascade of other defence responses (Keen, 1982; Gabriel and Rolfe, 1990).

In order to overcome this race-specific resistance the pathogen must evade recognition of its race-specific elicitor by the plant. Within plant pathogenic bacteria the evasion of recognition can be achieved in three different ways: (i) the avirulence gene may be mutated in such a way that its product is no longer responsible for the avirulent phenotype as is the case with *avrD* of *Pseudomonas syringae* pv. *glycinea* (Kobayashi *et al.*, 1990), (ii) the avirulence gene may be disrupted by a transposon as is the case with the avirulence gene *avrBs1* of *Xanthomonas campestris* pv. *vesicatoria* (Kearney *et al.*, 1988) and (iii) loss of the avirulence gene as described for *avrA* of *P. syringae* pv. *glycinea*, which is the most rigorous way of evasion of recognition by the host (Staskawicz *et al.*, 1984).

The outcome of different race-cultivar combinations is usually scored phenotypically, by development of disease symptoms (compatible interaction) or induction of HR (incompatible interaction), and is thought to be determined by specific host-pathogen recognition. The mechanism of recognition, however, is not known yet. Receptors in the host plasma membrane might interact with race-specific elicitors, followed by signal transduction and defence gene activation (Dixon and Lamb, 1990; Keen and Dawson, 1992; Scheel and Parker, 1990). The nature and origin of race-specific elicitors are poorly described. Many bacterial avirulence genes have been cloned, but their primary products do not induce HR (Knoop *et al.*, 1991; Ronald and Staskawicz, 1988; Staskawicz *et al.*, 1988). The primary product of *avrD* of *P. syringae* pv. *glycinea*, however, releases a lipid-like metabolite acting as a race-specific elicitor (Keen *et al.*, 1990; Keen and Buzzell, 1991).

Our work on the fungal pathogen *C. fulvum*, the causal agent of tomato leaf mould, has provided new insight into the mechanism of induction of HR by fungal avirulence gene products. Several race-specific elicitors, the putative products of avirulence genes, have been identified which induce necrosis on tomato cultivars with the corresponding resistance genes (De Wit and Spikman, 1982). One such race-specific peptide elicitor, the putative product

of the avirulence gene *avr*9, was purified from intercellular fluids (IF) of *C. fulvum*-infected tomato leaves (De Wit *et al.*, 1985; Scholtens-Toma and De Wit, 1988). The purified peptide elicitor specifically induces HR (observed as extensive necrosis) on tomato plants carrying the complementary resistance gene *Cf9*. The cDNA encoding the elicitor was isolated by using oligonucleotide probes derived from the amino acid sequence of the peptide (Van Kan *et al.*, 1991). The gene encoding the elicitor was only detected in fungal races which are avirulent on tomato genotypes carrying the resistance gene *Cf9*. Races virulent on *Cf9* genotypes of tomato which have evaded recognition by the host, do not produce the peptide elicitor (Scholtens-Toma *et al.*, 1989) as they lack the entire avirulence gene *avr*9 encoding this elicitor (Van Kan *et al.*, 1991).

In this study, we investigated the causal relationship between the presence of a functional *avr*9 gene and avirulence of races of *C. fulvum* on tomato genotype *Cf9*. We have isolated a genomic clone containing the avirulence gene *avr*9 , and used it to transform a race of *C. fulvum*, virulent on tomato genotype *Cf9*. All transformants became avirulent on tomato genotype *Cf9*, which clearly demonstrates that *avr*9 is a true avirulence gene, obeying the gene-for-gene hypothesis.

Results

Isolation and sequence analysis of the avr9 gene

A cDNA insert (A9-5) encoding the race-specific elicitor (Van Kan *et al.*, 1991) was used as a probe to screen 100,000 recombinant plaques of a genomic library of race 5 of *C. fulvum*. Several positive plaques were obtained and purified after a second round of screening. A restriction map of the DNA region surrounding the *avr*9 open reading frame (ORF) was obtained by Southern analysis of genomic DNA (Figure 1a). The map shows that a 3.8 kb *Xho*I fragment contains the ORF and approximately 3 kb of upstream sequences. Southern analysis of purified phage DNA indicated that one phage isolate contained the complete 3.8 kb *Xho*I fragment carrying the *avr*9 gene. The *Xho*I fragment was subcloned and from the resulting plasmid (pCF1) a more detailed restriction map was constructed (Figure 1b).

Sequence analysis revealed an open reading frame of 63 amino acids which is interrupted by one short intron of 59 bp (Figure 2). The 5' upstream region contains a putative TATA-box and 2 imperfect repeats of 61 bp which are 77% identical. The 3' untranslated region contains 8 direct repeats of 17 bp.

Transformation of C. fulvum

In order to prove that the cloned gene is responsible for the induction of genotype *Cf9*-specific resistance, transformation studies were performed. The *avr*9 gene was transferred to race 2.4.5.9.11 of *C. fulvum* (virulent on tomato genotype *Cf9*) by co-transformation with

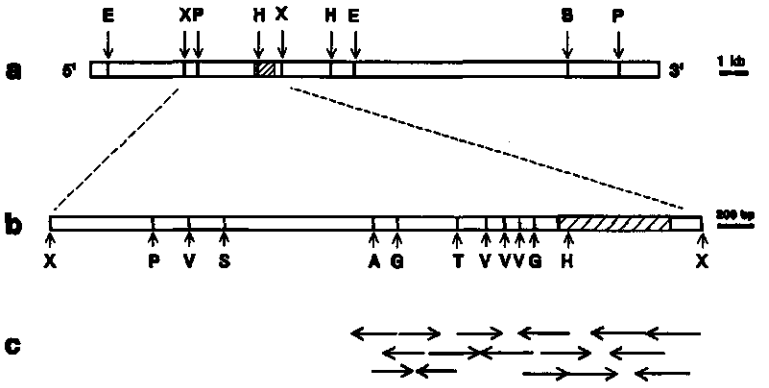

Figure 1. Restriction map of the DNA region surrounding the *avr*9 ORF. (a) Restriction map derived from Southern blot analysis of genomic DNA digested with several restriction enzymes and probed with the *avr*9 cDNA (hatched bar). (b) Restriction map of the subcloned 3.8 kb *Xho*I fragment containing the *avr*9 ORF (indicated by the hatched bar) and 3 kb of the promoter region. (c) The sequencing strategy is indicated by the arrows. A, *Xba*I; B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; T, *Sst*I; V, *Eco*RV; X, *Xho*I

pAN7-1, containing the hygromycin B resistance marker (*hph*). Six selected hygromycin B resistant (hygR) transformants were screened for integration of the *avr*9 gene by Southern blot analysis. Five out of six transformants (C1, C3 to C6) were found to have multiple copies of the *avr*9 gene integrated into their genomes, whereas one transformant (C2) had only obtained the *hph* marker (data not shown). Two transformants were selected and analysed in detail, C2 (HygR, avr9$^-$) and C3 (HygR, avr9$^+$).

Analysis of transformants C2 and C3

Southern blot analysis of transformants C2 and C3, and two wild type races of *C. fulvum* indicated that transformant C3 obtained several copies of the cloned *avr*9 gene (Figure 3, lane 4). The wild type *avr*9 gene, present in race 5, is located on a larger restriction fragment (lane 2). Wild type race 2.4.5.9.11, the recipient race (lane 1), and transformant C2 (lane 3) both lack the *avr*9 gene.

Figure 2 →. Sequence of the *Cladosporium fulvum* avirulence gene *avr*9 (EMBL accession number X60284). The gene encodes a precursor protein of 63 amino acids (Van Kan *et al.*, 1991) and is interrupted by a short 59 bp intron (----). The mature *avr*9 elicitor peptide of 28 amino acids is located at the carboxyl-terminus of the precursor protein (**bold**). A putative TATA-box (**TATAAGT**) is located 40 bp upstream of the main transcription start (⬇). The 5' upstream region contains 2 imperfect repeats of 61 bp (▭) that are 77% identical. The 3' untranscribed region contains 8 direct repeats of 17 bp (CGCATCGACTGCTCGGG, repeatedly over- and underlined).

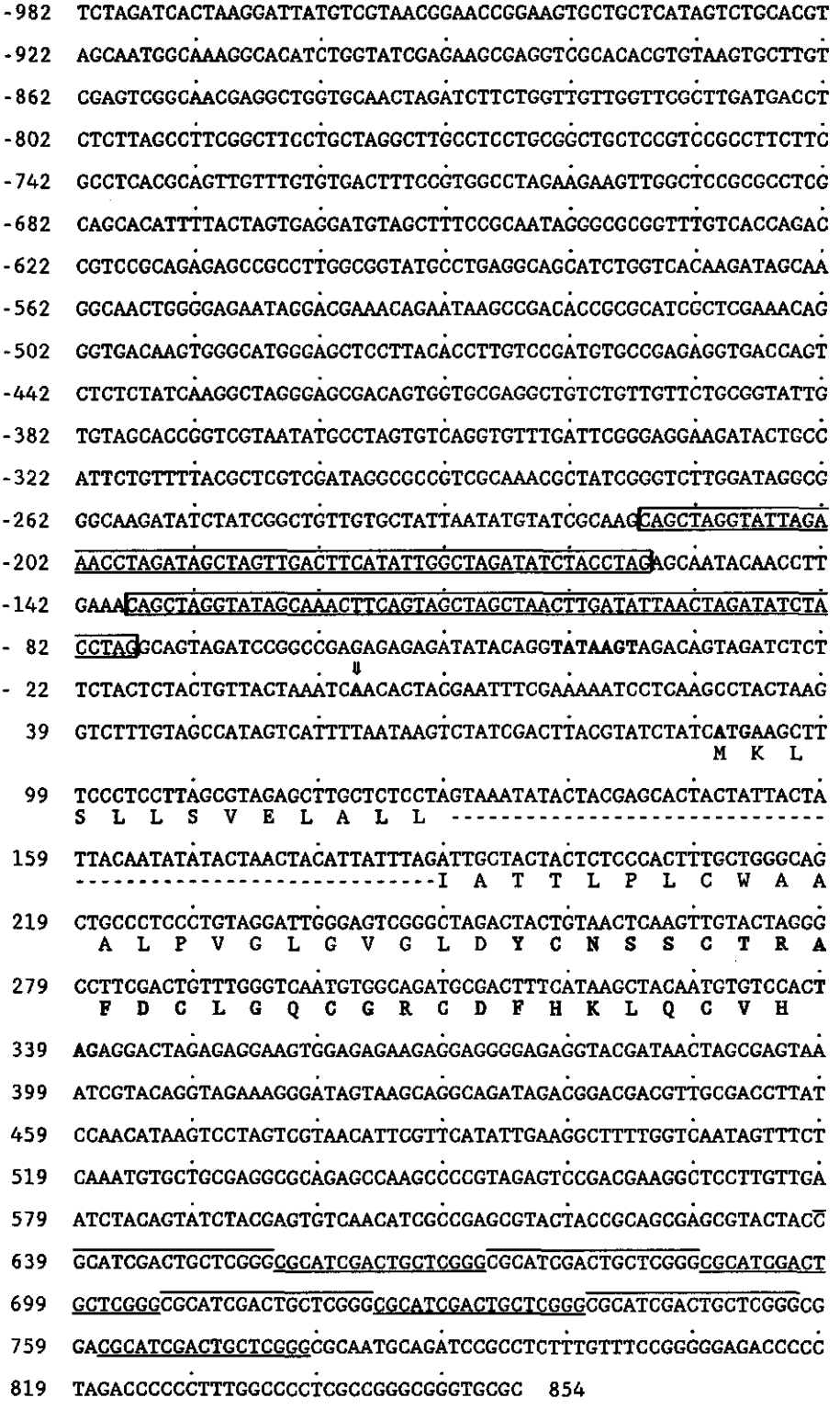
-982 TCTAGATCACTAAGGATTATGTCGTAACGGAACCGGAAGTGCTGCTCATAGTCTGCACGT
-922 AGCAATGGCAAAGGCACATCTGGTATCGAGAAGCGAGGTCGCACACGTGTAAGTGCTTGT
-862 CGAGTCGGCAACGAGGCTGGTGCAACTAGATCTTCTGGTTGTTGGTTCGCTTGATGACCT
-802 CTCTTAGCCTTCGGCTTCCTGCTAGGCTTGCCTCCTGCGGCTGCTCCGTCCGCCTTCTTC
-742 GCCTCACGCAGTTGTTTGTGTGACTTTCCGTGGCCTAGAAGAAGTTGGCTCCGCGCCTCG
-682 CAGCACATTTTACTAGTGAGGATGTAGCTTTCCGCAATAGGGCGCGGTTTGTCACCAGAC
-622 CGTCCGCAGAGAGCCGCCTTGGCGGTATGCCTGAGGCAGCATCTGGTCACAAGATAGCAA
-562 GGCAACTGGGGAGAATAGGACGAAACAGAATAAGCCGACACCGCGCATCGCTCGAAACAG
-502 GGTGACAAGTGGGCATGGGAGCTCCTTACACCTTGTCCGATGTGCCGAGAGGTGACCAGT
-442 CTCTCTATCAAGGCTAGGGAGCGACAGTGGTGCGAGGCTGTCTGTTGTTCTGCGGTATTG
-382 TGTAGCACCGGTCGTAATATGCCTAGTGTCAGGTGTTTGATTCGGGAGGAAGATACTGCC
-322 ATTCTGTTTTACGCTCGTCGATAGGCGCCGTCGCAAACGCTATCGGGTCTTGGATAGGCG
-262 GGCAAGATATCTATCGGCTGTTGTGCTATTAATATGTATCGCAAGCAGCTAGGTATTAGA
-202 AACCTAGATAGCTAGTTGACTTCATATTGGCTAGATATCTACCTAGAGCAATACAACCTT
-142 GAAACAGCTAGGTATAGCAAACTTCAGTAGCTAGCTAACTTGATATTAACTAGATATCTA
- 82 CCTAGGCAGTAGATCCGGCCGAGAGAGAGATATACAGGTATAAGTAGACAGTAGATCTCT
- 22 TCTACTCTACTGTTACTAAATCAACACTACGAATTTCGAAAAATCCTCAAGCCTACTAAG
39 GTCTTTGTAGCCATAGTCATTTTAATAAGTCTATCGACTTACGTATCTATCATGAAGCTT
M K L
99 TCCCTCCTTAGCGTAGAGCTTGCTCTCCTAGTAAATATACTACGAGCACTACTATTACTA
S L L S V E L A L L ----------
159 TTACAATATATACTAACTACATTATTTAGATTGCTACTACTCTCCCACTTTGCTGGGCAG
----------I A T T L P L C W A A
219 CTGCCCTCCCTGTAGGATTGGGAGTCGGGCTAGACTACTGTAACTCAAGTTGTACTAGGG
A L P V G L G V G L D Y C N S S C T R A
279 CCTTCGACTGTTTGGGTCAATGTGGCAGATGCGACTTTCATAAGCTACAATGTGTCCACT
F D C L G Q C G R C D F H K L Q C V H
339 AGAGGACTAGAGAGGAAGTGGAGAGAAGAGGAGGGGAGAGGTACGATAACTAGCGAGTAA
399 ATCGTACAGGTAGAAAGGGATAGTAAGCAGGCAGATAGACGGACGACGTTGCGACCTTAT
459 CCAACATAAGTCCTAGTCGTAACATTCGTTCATATTGAAGGCTTTTGGTCAATAGTTTCT
519 CAAATGTGCTGCGAGGCGCAGAGCCAAGCCCCGTAGAGTCCGACGAAGGCTCCTTGTTGA
579 ATCTACAGTATCTACGAGTGTCAACATCGCCGAGCGTACTACCGCAGCGAGCGTACTACC
639 GCATCGACTGCTCGGGCGCATCGACTGCTCGGGCGCATCGACTGCTCGGGCGCATCGACT
699 GCTCGGGCGCATCGACTGCTCGGGCGCATCGACTGCTCGGGCGCATCGACTGCTCGGGCG
759 GACGCATCGACTGCTCGGGCGCAATGCAGATCCGCCTCTTTGTTTCCGGGGGAGACCCCC
819 TAGACCCCCCTTTGGCCCCTCGCCGGGCGGGTGCGC 854

Table 1. Pathogenicity of transformants C2 and C3 on cultivar MM (lacking *Cf*-resistance genes) and genotype *Cf9* (carrying resistance gene *Cf9*).

Transformant	cultivar MM	genotype *Cf9*
C2	C	C
C3	C	I

C: Compatible interaction; race virulent; fungal growth and sporulation. I: Incompatible; race avirulent; no fungal growth.

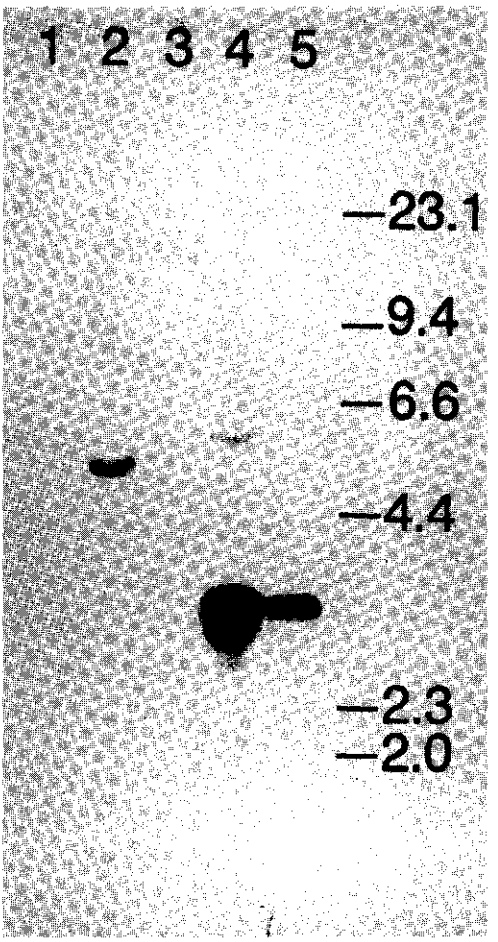

Figure 3. Southern blot analysis of genomic DNA from wild type *C. fulvum* races and transformants digested with *Pst*I and *Eco*RI. Lane 1, wild type race 2.4.5.9.11, the recipient race, virulent on genotype *Cf9*; lane 2, wild type race 5, avirulent on genotype *Cf9*; lane 3 & 4, transformants C2 and C3 respectively.; lane 5, plasmid pCF1 containing the *avr9* gene. Race 5 (lane 2) contains the wild type gene on a 5 kb *Pst*I/*Eco*RI fragment, whereas transformant C3 (lane 4) contains the introduced *avr9* gene on a 3 kb *Pst*I fragment (due to a *Pst*I site in the polylinker of the vector). Race 2.4.5.9.11 (lane 1) and transformant C2 (lane 3) lack the *avr9* gene.

Pathogenicity of transformants C2 and C3 was tested on tomato seedlings of cultivar Moneymaker (MM, susceptible to all races of *C. fulvum*, carrying no *Cf*-resistance genes) and genotype *Cf9* (a near-isogenic line of cultivar Moneymaker carrying resistance gene *Cf9*). Symptoms were visible 14-20 days after inoculation (Table 1). Transformants C2 and C3 were both still virulent on cultivar MM, indicating that pathogenicity was not affected by the transformation procedure. The pathogenicity of transformant C3 on genotype *Cf9*, however, was completely changed from virulent to avirulent, whereas transformant C2 remained virulent.

To confirm the observed difference in growth of the virulent transformant C2 and avirulent transformant C3, total DNA was isolated from the leaves infected with both transformants and the presence of fungal DNA was semi-quantified by using a single copy probe of *C. fulvum* (Figure 4). The signals on the Southern blot confirm the phenotypic observations. Visually compatible interactions (C2/MM, C2/*Cf9* and C3/MM) gave a clear

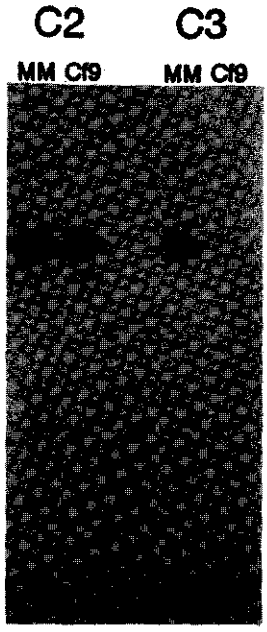

Figure 4. Southern blot analysis of total DNA isolated from infected leaves, probed with the single copy *C. fulvum* gene *ecp*1 (Van den Ackerveken *et al.*, 1993). The hybridization signal (a 1 kb *Hin*dIII fragment) reflects the quantity of fungal biomass in the infected leaves. Hybridization was only detected in those interactions which were visually scored as compatible (C2/MM, C2/*Cf9* and C3/MM) but not in those scored as incompatible (C3/*Cf9*).

hybridization signal, indicating that a significant proportion from the DNA of the infected leaves is of fungal origin. The leaves of C3-infected genotype *Cf9* did not contain a detectable amount of fungal DNA, indicating the absence of fungal growth.

The cultivar-specificity of transformant C3 has changed from virulent to avirulent on tomato genotype *Cf9*. Accordingly the elicitor encoded by *avr*9, which induces HR on *Cf9* genotypes but not on MM, should be produced by transformant C3. Intercellular fluid (IF) isolated from both compatible interactions C3/MM and C2/MM was screened for the presence of the necrosis-inducing peptide elicitor. Necrosis-inducing activity was assayed by injection of IF into tomato genotype *Cf9* and cultivar MM. IF from the interaction C3/MM contained high necrosis-inducing activity, indicating that active *avr*9 elicitor is indeed produced by this transformant. Transformant C2 did not produce any *avr*9 elicitor (Figure 5). The other four transformants carrying *avr*9 (C1, C4, C5 and C6) also appeared to be avirulent on tomato genotype *Cf9* and accordingly produced active *avr*9 elicitor (results not presented).

These results clearly indicate that the cloned avirulence gene *avr*9 is responsible for the specific induction of resistance on genotype *Cf9*, and that no other genes or factors are required to restore avirulence in a race previously virulent on tomato genotype *Cf9*.

Discussion

Recognition of the pathogen by the host, the basis of resistance and susceptibility in gene-for-gene relationships, is poorly understood. Much effort has been put into unraveling induced defence responses in resistant plants, but the mechanisms of induction are still largely unknown (Bowles, 1990; Lamb *et al.*, 1989; Scheel and Parker, 1990). The genetics of fungal avirulence and cultivar-specific resistance are generally well studied (De Wit, 1992; Ellingboe, 1981; Flor, 1971; Ilott *et al.*, 1989). In most cases, however, the direct or indirect products of avirulence genes and resistance genes are not characterized.

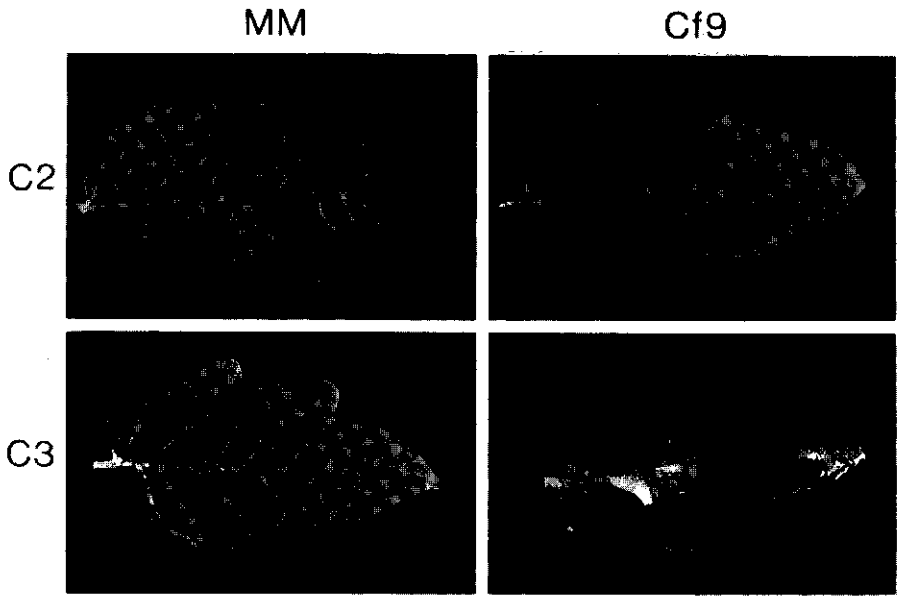

Figure 5. Necrosis-inducing activity of intercellular fluid (IF) isolated from C2- and C3-infected Moneymaker (MM) leaves (compatible interactions). The intensity of necrosis in genotype *Cf9* is correlated with the amount of injected peptide elicitor, encoded by the *avr9* gene. Necrosis-inducing activity was found in IF of C3-infected leaves, indicating that the elicitor produced by the transformant is active.

Our approach to elucidate the mechanism of recognition in the *C. fulvum*-tomato system has resulted in the cloning of *avr9*, the first fungal avirulence gene to be cloned.

We were able to clone the *avr9* gene by using amino acid sequence data from the race-specific elicitor peptide (Scholtens-Toma and De Wit, 1988). A degenerated oligonucleotide probe was employed to isolate the *avr9* cDNA clone (Van Kan *et al.*, 1991), which was subsequently used to isolate the *avr9* gene from a genomic library of race 5 of *C. fulvum*, a fungal race avirulent on tomato genotype *Cf9*. Sequence analysis revealed that the ORF is interrupted by a 59 bp intron, which is common for fungal genes (Gurr *et al.*, 1987). The possible role of the repeats in the 5' upstream region and putative TATA-box in the regulation of gene expression will be studied by promoter analysis. The significance of the repeats present in the 3' end of the gene, but are absent in the mature mRNA, is unclear.

To demonstrate the causal relationship between a functional *avr9* gene encoding the elicitor and avirulence on tomato genotype *Cf9*, the cloned *avr9* gene was transferred to race 2.4.5.9.11 of *C. fulvum*, which lacks the *avr9* gene and can overcome the resistance genes *Cf2*, *Cf4*, *Cf5*, *Cf9* and *Cf11* of tomato. The cultivar-specificity of the transformants containing the *avr9* gene changed from virulent to avirulent on tomato genotype *Cf9*. The elicitor encoded by the *avr9* gene could be detected in IF of compatible interactions, demonstrating significant expression of the transferred *avr9* gene in these transformants.

Many bacterial avirulence genes have been cloned by supplementing recipient virulent strains with DNA from an avirulent strain, followed by screening for avirulence on appropriate cultivars (Keen and Staskawicz, 1988; Staskawicz *et al.*, 1984; Staskawicz *et al.*, 1988; Swanson *et al.*, 1988). The primary products of these bacterial avirulence genes, however, are not directly responsible for the induction of HR in cultivars carrying the corresponding resistance genes. The primary gene products of for instance *avrA*, *avrB* and *avrC* from *Pseudomonas syringae* pv. *glycinea* lack a leader peptide secretion sequence and significant hydrophobic domains, suggesting that these proteins are not excreted (Keen and Staskawicz, 1988). The primary product of *avrBs3* of *X. campestris* pv. *vesicatoria* seems to be soluble but is localized intracellularly (Knoop *et al.*, 1991). The product of the *avrD* gene from *P. syringae* pv. *tomato* has been shown to act via the release of low molecular weight lipid-like elicitors originating from bacterial substrates (Keen *et al.*, 1990). Our studies on the race-specific *avr9* elicitor peptide is the the first example of a primary avirulence gene product, which directly induces HR in cultivars carrying the matching resistance gene.

Further support for the elicitor-receptor model for the *avr9-Cf9* system can be obtained by cloning the *Cf9* resistance gene. It is tempting to speculate about the existence of a receptor (the putative product of the resistance gene *Cf9*) for the *avr9* elicitor in tomato. The only example of a putative receptor is demonstrated in soybean for the heptaglucoside elicitor from *Phytophthora megasperma* (Cosio *et al.*, 1990; Yoshikawa *et al.*, 1983). This elicitor was obtained by acid hydrolysis of fungal cell walls and is not involved in race-cultivar specific resistance. In contrast, the *avr9* peptide is a naturally occuring race-specific elicitor and its specific and rapid induction of HR on tomato genotype *Cf9* strongly suggests the presence of a specific receptor. The putative plasma membrane receptor for the *avr9* peptide elicitor might be the primary product of the *Cf9* resistance gene.

The biological function of the *avr9* peptide for *C. fulvum*, while growing on a susceptible plant, is still unknown. The *avr9* gene is highly expressed when the fungus grows in the plant (Van Kan *et al.*, 1991) suggesting that the peptide is of physiological importance for the pathogen. Races of *C. fulvum* that have overcome resistance gene *Cf9* of tomato by loss of the *avr9* gene are still pathogenic, indicating that the *avr9* gene is dispensible. Nevertheless, the *Cf9* resistance gene still provides a good protection to *C. fulvum* in greenhouse-grown tomato cultivars, suggesting that the competitive ability of these new races is slightly reduced. To quantify the role of the *avr9* elicitor in fitness, the presence or absence of a functional *avr9* gene in the population should be studied in competition experiments.

Experimental procedures

Subculture of C. fulvum and pathogenicity tests

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). The races

used and their interaction with different tomato genotypes are presented in Table 2. Pathogenicity was tested by inoculation of 14-day-old tomato seedlings at the lower side of the cotyledon with a conidial suspension of $1x10^5$ spores ml^{-1} (Talbot *et al.*, 1988). Symptoms were apparent 14-20 days after inoculation. In a compatible interaction, fungal growth was observed as sporulation on the leaf surface, whereas in the incompatible interaction (resistant plant) these symptoms were not visible.

Table 2. Races of *C. fulvum* used in this study. The interaction with two cultivars of tomato, production of *avr9* elicitor and response of the cultivars to the elicitor are indicated.

Race	Proposed genotype	Cultivars		*avr9* elicitor	HR induction	
		MM	*Cf9*		MM	*Cf9*
5	A2A4a5A9A11	C	I	yes	no	yes
2.4.5.9.11	a2a4a5a9a11	C	C	no	no	no

C: Compatible interaction; race virulent; fungal growth and sporulation.
I: Incompatible; race avirulent; no fungal growth.

Isolations of genomic DNA and construction of a genomic library

DNA was isolated from liquid-grown cultures as previously described (Van Kan *et al.*, 1991). Total DNA of race 5 of *C. fulvum* was partially digested with *Sau*3AI and sized by sucrose gradient centrifugation (10-40 %). Fragments of 10-25 kb were ligated into *Bam*HI digested lambda EMBL3 arms (Promega, Madison, WI). Following ligation, lambda phage DNA was packaged with a Packagene kit (Promega) and plated on *Escherichia coli* MB406. The primary library (800,000 independent recombinants) was amplified and stored at 4°C.

Isolation of the avr9 gene, subcloning and sequencing

All DNA manipulations were conducted essentially as described by Maniatis *et al.* (1982). The primary genomic library was plated on *E. coli* MB406. Two replica filters were made on Hybond N (Amersham Nederland BV, Houten, The Netherlands) from two plates containing 100,000 independent recombinants, according to the instructions of the manufacturer. The filters were hybridized in 5xSSC, 5xDenhardt's, 0.5% SDS and 100 $\mu g.ml^{-1}$ denatured calf thymus DNA at 65°C, using as probe a 400 bp *avr9* cDNA insert (A9-5, Van Kan *et al.*, 1991), which was radioactively labeled with [α-^{32}P]dATP by the random primer method (Hodgson and Fisk, 1987). Selected lambda clones were analysed by digestion with several restriction enzymes and Southern hybridization with the *avr9* cDNA-probe. A 3.8 kb *Xho*I fragment was subcloned in *Sal*I digested plasmid pGEM 3Zf+ (Promega), resulting in plasmid pCF1. The 3' region of the *Xho*I fragment, containing the ORF and promoter region, was sequenced by using the dideoxy chain termination method (Sanger *et al.*, 1977) on double-stranded DNA (Promega).

Fungal protoplast isolation and fungal transformation

Protoplasts were isolated from 48-hour-old mycelium as decribed by Harling *et al.* (1988) with the modification that liquid B5-medium was used instead of potato dextrose broth. The *avr*9 gene was transferred to *C. fulvum* race 2.4.5.9.11 by co-transformation with pAN7-1 containing a hygromycin B resistance marker (Punt *et al.*, 1987). Two μg of pAN7-1 and 4 μg of pCF1 were mixed with $1x10^6$ protoplasts and treated with PEG as described previously (Oliver *et al.*, 1987). The protoplast mixture was diluted with liquid complete medium and plated in top agar onto stabilized regeneration medium (Harling *et al.*, 1988). Twenty hours after plating, a second top-layer was applied containing 800 $\mu g.ml^{-1}$ hygromycin B (final concentration of 100 $\mu g.ml^{-1}$). After approximately 4 weeks single spore cultures were made and individual transformants were analysed.

DNA analysis of transformants and infected tomato leaves

DNA of liquid-grown cultures of transformants and wild type races of *C. fulvum* was isolated as previously described (Van Kan *et al.*, 1991), digested with *Eco*RI and *Pst*I, electrophoresed in 0.8% agarose, transferred to Hybond-N^+ by vacuum blotting (Millipore BV, Etten Leur, The Netherlands) and probed with a random primed [α-^{32}P]dATP-labeled 0.4 kb *avr*9 cDNA fragment.

Fourteen days after inoculation of tomato seedlings, leaves were collected and freeze-dried. Genomic DNA was isolated from the leaves, digested with *Eco*RI and *Hin*dIII, electrophoresed in 0.8% agarose, transferred to Hybond-N^+ by vacuum blotting and probed with a random primed [α-^{32}P]dATP-labeled 1 kb *Hin*dIII fragment containing the single copy *C. fulvum* gene encoding the protein ECP1 (Joosten and De Wit, 1988; Van den Ackerveken *et al.*, 1993).

Intercellular fluid isolation and elicitor activity bioassay.

For testing elicitor activity, the intercellular fluid (IF) of leaves from the pathogenicity tests was isolated. Leaves were infiltrated with distilled water *in vacuo* and IF was isolated by centrifugation as described previously (De Wit and Spikman, 1982). Fifty μl of IF was injected into leaves of cultivar MM (no *Cf*-genes) and genotype *Cf9* (carrying resistance gene *Cf9*). One to two days after injection, the elicitor activity was visible as necrosis (HR) on genotype *Cf9* but not on cultivar MM.

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Chapter 4

Disruption of the avirulence gene *avr9* in *Cladosporium fulvum* causes virulence on tomato genotypes with the complementary resistance gene *Cf9*

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Summary

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

Introduction

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

For our model species *Cladosporium fulvum*, a biotrophic fungal pathogen of tomato, no sexual stage has ever been described and the existence of six avirulence genes has been postulated based on the differential interactions of wild-type fungal races on tomato cultivars known to possess different resistance genes toward this fungus. The genetic basis of the gene-for-gene interaction between tomato and *C. fulvum* has therefore only been established clearly for the host plant tomato for which the different *Cf* resistance genes have now been positioned on the genetic map (Jones *et al.*, 1991; Van der Beek *et al.*, 1992). It has also been shown that avirulent races of the fungus produce proteinaceous elicitors inducing a necrotic reaction only when injected into the leaves of the corresponding resistant plant (De Wit and Spikman, 1982). Because of the specificities of these elicitors, whose necrosis-inducing activities are reminiscent of the hypersensitive responses observed following the infection of a resistant plant by an avirulent fungal race, it was suggested that they could be the primary products of the avirulence genes. The necrosis-inducing peptide produced by fungal races avirulent on tomato genotype *Cf9* (avr9$^+$ strains) was purified and sequenced (Scholtens-Toma and De Wit, 1988). The sequence data from this peptide were used to design oligonucleotides which were employed to isolate the cDNA and genomic clones encoding the AVR9 peptide (Van Kan *et al.*, 1991; Van den Ackerveken *et al.*, 1992). These authors showed that the genomic coding region is interrupted by one intron and that the primary gene product is a 63 amino-acid-long peptide, from which only the 28 C-terminal part has been found in infected tomato leaves. Southern hybridization experiments established the presence of this gene in the genomes of all the avr9$^+$ fungal races tested but failed to detect homologous sequences in the genomes of the two races virulent on tomato genotype *Cf9* tested, suggesting that these isolates could have arisen following a deletion in this genomic region. Karyotype analysis indicated that races 2.4.9.11 and 2.4.5.9.11, both lacking *avr9*, have undergone an estimated deletion of 500 kb from chromosome band 2, suggesting that these virulent races might have lost functional chromosomal DNA in addition to the *avr9* gene (Talbot *et al.*, 1991). Transcripts of the *avr9* gene could only be detected in susceptible host plant infected by avr9$^+$ races. The genomic sequence could be introduced

by transformation and expressed in avr9$^-$ strains, changing them from virulent to avirulent on tomato genotype *Cf9*. These results clearly demonstrated that the AVR9 peptide is sufficient for triggering the hypersensitive response in combination with the plant resistance gene *Cf9* supporting therefore the gene-for-gene hypothesis.

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

Results

Isolation and characterization of uridine auxotrophic mutants

In order to improve the methodology for the genetic manipulation of *C. fulvum* we tried to isolate mutants which could be used for transformation with either homologous or heterologous genes. For each of the two wild-type races (races 4 and 5, both containing avirulence gene *avr9*), we selected 20 mutants resistant to FOA following UV mutagenesis. Among them one from race 4 and four from race 5 were uridine auxotrophs. It was shown that in yeast (Boeke *et al.*, 1984) the mutants isolated using this method could result from mutations in either the gene encoding the orotidine-5'-phosphate decarboxylase (OMPdecase) or the gene coding for the orotate pyrophosphoribosyl transferase (OPRTase). To decide between these two possibilities, we transformed each of the *C. fulvum* mutants with the cloned *pyr*A gene from *Aspergillus niger* coding for the OMPdecase (Goosen *et al.*, 1987). Three of the race 5 mutants and the race 4 one could be efficiently complemented by this heterologous gene and Southern blot analyses indeed confirmed the presence of the *A. niger* gene integrated in their genomes (data not shown). From this result we concluded that these four mutants have a mutation in the structural gene for the OMPdecase and that the fifth one which we could not complement might have a mutation in the gene coding for the OPRTase. Mutants 5.4 from race 5 and 4.2 from race 4 were used as recipient strains for the disruption of the *avr9* gene.

Construction of a plasmid for the disruption of the avr9 gene and transformations

We designed a plasmid allowing a single step gene replacement at the *avr9* locus. The *avr9* coding region present on the lambda clone described by Van den Ackerveken *et al.* (1992) was subcloned as a 6 kb *Eco*RI-*Sal*I fragment into pBluescript (Stratagene). An internal 750 bp *Hin*dIII-*Xho*I fragment containing the whole coding sequence was removed and replaced by the *A. nidulans pyr*G gene present on a 1.5 kb DNA fragment. Figure 1 gives details of

the construction of this pCF112 plasmid. The presence of the *pyr*G gene, coding for an OMPdecase, allows the transformation of *C. fulvum* pyr⁻ mutants to prototrophy. Upstream and downstream of this *pyr*G gene are 2.5 and 2.7 kb of *C. fulvum* DNA sequences which could allow the replacement of the *avr*9 gene following homologous recombination events as illustrated in Figure 1.

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

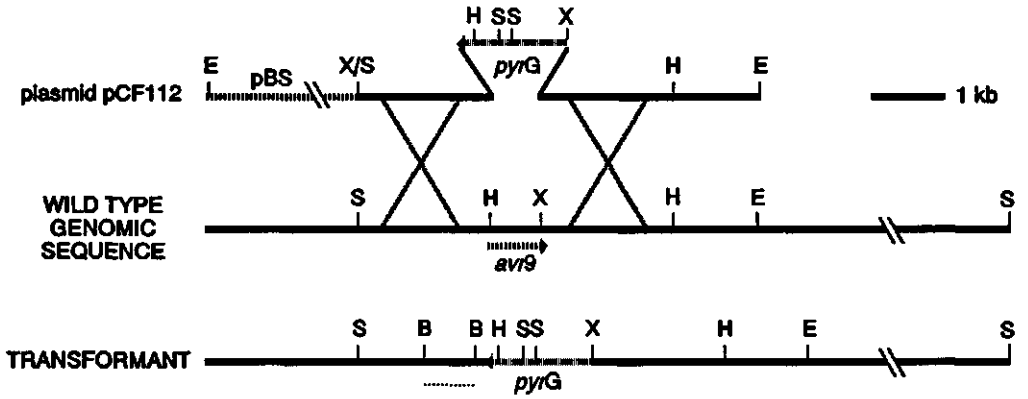

Figure 1. Construction of the plasmid pCF112 used to disrupt the *avr*9 gene and events leading to the replacement of the gene following transformation. The *avr*9 genomic clone was obtained as a 6 kb *Eco*RI-*Sal*I fragment cloned into the *Eco*RI-*Xho*I sites of pBluescript. A *Hin*dIII site close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

Characterization of avr9⁻ transformants

Genomic DNAs extracted from the transformants were initially subjected to dot blot analysis using the 750 bp *avr9* coding sequence, removed from the transforming plasmid, as the hybridization probe. The DNA from three transformants (one from race 4 and two from race 5) failed to give any hybridization signal (results not shown) and one such transformant from each race (race 4-A43 and race 5-B51) was subjected to detailed Southern blot analyses along with the DNAs from the wild-type race 5 and from two other transformants (race 4-A11 and race 5-B11) with ectopic integrations of the plasmid. The hybridization to the labeled *avr9* coding sequence was repeated on *Hin*dIII digested genomic DNAs (Figure 2, panel A). This probe hybridized to a 2.3 kb DNA fragment of the wild-type race 5 and transformants A11 and B11 but did not hybridize to the DNAs from transformants A43 and B51 (note that a polymorphism exists between races 4 and 5, seen as a slightly larger hybridizing fragment in the race 4-A11 transformant). [α-^{32}P]dATP-labeled pBluescript sequences only hybridized to the DNAs of transformants A11 and B11 which originated from non-homologous integrations of the plasmid (Figure 2, panel B). When a 800 bp *Bgl*II fragment upstream of the *avr9* gene (Figure 1) was used to probe *Sal*I digested DNAs, it identified a 10 kb DNA fragment in the genome of the untransformed race 4 and one additional larger fragment in transformants A11 and B11 (Figure 2, panel C; note that also in this case a DNA polymorphism between the transformants is visible). By contrast, this same probe identified a unique 3 kb fragment in the genomes of tranformants A43 and B51 (Figure 2, panel C); the size of this last fragment is in agreement with the insertion of the *pyr*G gene, which contains two internal *Sal*I sites.

From these analyses we concluded that transformants A43 and B51 have both arisen from a single step gene replacement at the *avr9* locus which has been exchanged by the *pyr*G gene present on the transforming pCF112 plasmid. No other integrations of the plasmid at ectopic sites seemed to have occurred in those two transformants. Transformants A11 and B11, containing several ectopic integrations of pCF112, were used as controls to assess the effect of transformation on pathogenicity.

Pathogenicity and virulence of the transformants

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

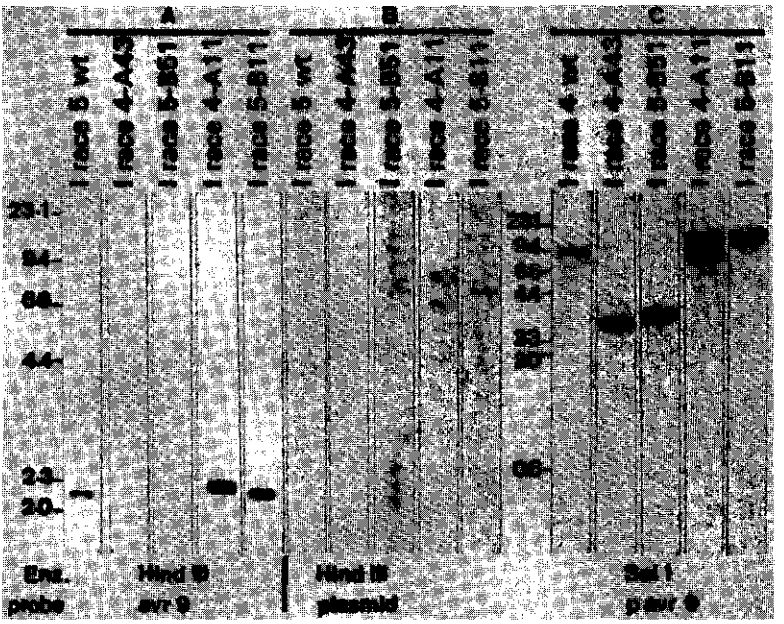

Figure 2. Southern blot analyses demonstrating that transformants A43 and B51 result from a gene replacement at the *avr*9 locus. The restriction enzymes used to digest the genomic DNAs and the probes used are indicated below the autoradiographs. The hybridization patterns of transformants A43 and B51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

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

The ability of the avr$^-$ transformants to infect tomato genotype *Cf9* was not simply a consequence of the transformation since transformants A11 and B11 which have ectopic integrations of the pCF112 plasmid retained the host specificities of the wild-type races 4 and 5, being able to infect MM plants only.

The production of an active elicitor peptide was proven by injecting intercellular fluids collected from the different interactions into the leaves of MM and tomato genotype *Cf9*. As summarised in Table 1, a necrotic response could only be detected when intercellular fluids

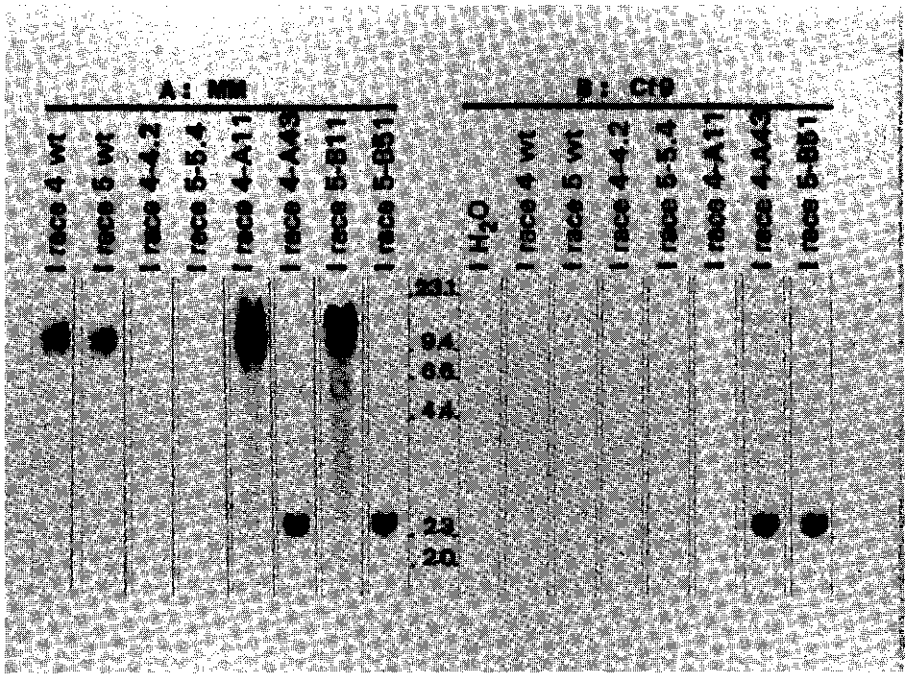

Figure 3. Southern blot analysis of DNAs extracted from the leaves of infected tomato plants 14 days after inoculation. Genomic DNAs (from both plant and fungal origins) were digested with *SalI* and the membrane was probed with the *BglII* fragment upstream of the *avr9* gene (as in Fig. 2C). Each lane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

from avr9$^+$/MM interactions were injected into the leaves of genotype *Cf9*, while transformants A43 and B51 did not produce any elicitor. Necrotic reactions were observed even when intercellular fluids of avr9$^+$/MM interactions were diluted 10 fold, while undiluted samples of avr9$^-$/MM, *Cf9* interactions failed to induce necrosis.

These results clearly demonstrate that the *avr9* gene, present in the races 4 and 5 of *C. fulvum*, is the only genetic factor responsible for their inability to infect tomato genotype *Cf9*. They also indicate that the AVR9 peptide is the only compound from crude intercellular fluids which induces necrosis in tomato genotype *Cf9*.

Table 1. Characteristics of the *C. fulvum* strains used in this study

Strain	Description	Cultivar MM	Cultivar *Cf9*	production of AVR9 elicitor
race 5	wild-type isolate	C	I	+
race 5-5.4	pyr⁻ mutant induced in race 5 by UV mutagenesis	*	*	
race 5-B51	race 5-5.4 *avr9*: :*pyrG* insertion mutant	C	C	-
race 5-B11	race 5-5.4 with the *avr9*: :*pyrG* construct at ectopic sites	C	I	+
race 4	wild-type isolate	C	I	+
race 4-4.2	pyr⁻ mutant induced in race 4 by UV mutagenesis	*	*	
race 4-A43	race 4-4.2 *avr9*: :*pyrG* insertion mutant	C	C	-
race 4-A11	race 4-4.2 with the *avr9*: :*pyrG* construct at ectopic sites	C	I	+

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

Discussion

In this paper we demonstrate that a single mutation generated by gene disruption can alter the cultivar-specificity of the fungal pathogen *C. fulvum*. In the case of the *avr9* gene we recovered three transformants which had resulted from a gene replacement out of the 95 we analyzed, which corresponds to a frequency in the range of 1-5%. The data presented confirm that gene disruption by gene replacement is possible in the filamentous plant pathogenic fungus *C. fulvum*, and provides new possibilities for the molecular genetic analysis of avirulence and pathogenicity. Although gene disruption occurs at a low frequency, the use of this method could be extended in the future to assess the function of other *C. fulvum* genes whose products are found in intercellular fluids from infected plants and which are putative pathogenicity factors (Joosten and De Wit, 1988; Van den Ackerveken *et al.*,

1993). Successful gene replacement has been described for a number of other plant pathogenic fungi including *Ustilago maydis* (Fotheringham and Holloman, 1989; Kronstad *et al.*, 1989), *Cochliobolus carbonum* (Scott-Craig *et al.*, 1990), *Nectria haematococca* (Stahl and Schäfer, 1992), *Magnaporthe grisea* (Sweigard *et al.*, 1992) and *Gibberella pulicaris* (Hohn *et al.*, 1992).

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

At the start of this study, we knew from the experiments performed by Van den Ackerveken *et al.* (1992) that the transfer of the *avr9* sequence to a virulent race of *C. fulvum* was sufficient to make it avirulent on tomato genotype *Cf9*. From the results presented in this paper it becomes clear that in the two wild-type races we studied, the *avr9* gene is indeed the only genetic factor responsible for the induction of an incompatible interaction on tomato genotype *Cf9*. This definitively establishes the genetic basis of the gene-for-gene relationship in the case of the *avr9*/*Cf9* interaction and also confirms the direct involvement of the AVR9 peptide in the development of the hypersensitive response. The *avr9* gene is the only avirulence gene cloned so far for which the processed protein product has been shown to be directly responsible for the induction of the hypersensitive response. This has not been established or does not seem to be the case for most of the bacterial avirulence genes which have been characterized; there is no evidence for their translation products to be excreted and small molecules, generated by the avirulence gene products, seem to act as race-specific signals as in the case of the *avrD* gene from *Pseudomonas syringae* pv. tomato (Keen *et al.*, 1990). It remains to be proven whether other *C. fulvum* race-specific elicitors described by De Wit and Spikman (1982) are the direct avirulence gene products as well.

The simple organization of the *avr9* locus which consists of a single open reading frame supports the idea that avr9$^-$ field isolates could have arisen from a single mutation in a previously avr9$^+$ genetic background. Data from Van Kan *et al.* (1991) indeed showed that in two field isolates virulent on tomato genotype *Cf9* the *avr9* gene was deleted. Karyotype analysis of different races of *C. fulvum* revealed that wild-type races 2.4.9.11 and 2.4.5.9.11, both lacking *avr9*, have a deletion of 500 kb. (Talbot *et al.* 1991). Recent data indicate that the deletion is somewhat smaller (R. Oliver, personal communication; G. Van den Ackerveken, unpublished). A similar situation has recently been reported by Panaccione *et al.* (1992) who showed that the *TOX2* locus of the maize pathogen *Cochliobolus carbonum*,

which is involved in the first step of the synthesis of the cultivar specific HC-toxin, is not found in the genomes of toxin nonproducing (tox$^-$) strains. In a recent study, Miao *et al.* (1991) demonstrated that the *Pda6* gene from *Nectria haematococca* (encoding a cytochrome P-450 enzyme responsible for the detoxification of the pea phytoalexin pisatin) was on a dispensable B chromosome which did not segregate during meiosis, giving more pda$^-$ progeny than expected.

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

Experimental procedures

Fungal strains, mutagenesis and growth conditions

The *C. fulvum* (syn. *Fulvia fulva*) strains used in this study are listed in Table 1. The pyrimidine auxotrophic mutants were selected among FOA resistant mutants as originally described for yeast by Boeke *et al.* (1984). Conidial suspensions in water were plated onto minimal medium supplemented with 10 mM of uridine and 2.5 (race 5) or 3 (race 4) mg.ml^{-1} FOA; after UV irradiation to give 30-40% survival, the plates were kept in the dark for two

weeks. FOA resistant mutants were purified onto fresh FOA plates and subsequently analyzed for uridine auxotrophy.

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

Plant material and inoculations

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

Fungal transformation

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

DNA manipulations

Standard DNA techniques used were as described by Sambrook *et al.* (1989). The *E. coli* strain DH5α (F^- ϕ80d*lac*Z M15 (*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17($r_k^-m_{k+}$) *deo*R *thi*-1 *sup*E44 *gyr*A96 *rel*A1) was used to propagate plasmids. *C. fulvum* genomic DNA was extracted from freeze-dried mycelia using the method described by Van Kan *et al.* (1991). DNA from infected leaves was extracted as described by Van der Beek *et al.* (1992). Following restriction-enzyme digests, DNA fragments were separated in 0.7% agarose gels and transferred by Southern blotting onto Hybond N^+ membranes using 0.4 M NaOH according to the manufacturer's instructions (Amersham). Membranes were probed with random-primed [α-^{32}P]dATP-labeled DNA fragments, hybridizations and post-hybridization washes were carried out at 65°C in aqueous buffers.

Acknowledgements

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Chapter 5

The AVR9 race-specific elicitor of *Cladosporium fulvum* is processed by endogenous and plant proteases

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adapted from *Plant Physiology 103 (1993), In press*

Summary

The avirulence gene *avr*9 of the fungal tomato pathogen *Cladosporium fulvum* encodes a race-specific peptide elicitor, that induces a hypersensitive response in tomato plants carrying the complementary resistance gene *Cf9*. The *avr*9 gene is highly expressed when *C. fulvum* is growing *in planta* and the elicitor accumulates in infected leaves as a 28 aa peptide. In *C. fulvum* grown *in vitro*, the peptide elicitor is not produced in detectable amounts. In order to produce significant amounts of the AVR9 elicitor *in vitro*, the coding and termination sequences of the *avr*9 gene were fused to the constitutive *gpd*-promoter (glyceraldehyde-3-phosphate dehydrogenase) of *Aspergillus nidulans*. Transformants of *C. fulvum* were obtained that highly expressed the *avr*9 gene *in vitro* and produced active AVR9 peptide elicitors. These peptides were partially sequenced from the N-terminus and appeared to consist of 32, 33 and 34 aa, respectively and are the precursors of the mature 28 aa AVR9 peptide. We demonstrated that plant factors process the 34 aa peptide into the mature 28 aa peptide. We present a model for the processing of AVR9 involving cleavage of a signal peptide during excretion and further maturation by fungal and plant proteases into the stable 28 aa peptide elicitor.

Introduction

Specific recognition in the gene-for-gene interaction between the fungal pathogen *Cladosporium fulvum* and tomato is mediated via so-called race-specific elicitors, the primary products of avirulence genes (Van Kan *et al.*, 1991; Van den Ackerveken *et al.*, 1992). Receptors in the resistant plant, which might be the direct products of resistance genes, are thought to recognize the specific elicitors (De Wit, 1992). The subsequent defence reaction is characterized by a hypersensitive response (HR) which restricts growth of the pathogen to the site of penetration.

Race-specific elicitors in the *C. fulvum* - tomato interaction were first identified by De Wit and Spikman (1982). One such race-specific elicitor, inducing HR on tomato plants carrying the resistance gene *Cf9*, was purified and characterized (De Wit *et al.*, 1985) and the amino acid sequence was determined (Scholtens-Toma and De Wit, 1988). The corresponding cDNA was isolated using oligonucleotide probes and designated avirulence gene *avr9* (Van Kan *et al.*, 1991). Races of *C. fulvum* virulent on tomato genotype *Cf9* completely lack the *avr9* gene and therefore do not produce AVR9 peptide elicitor. Transformation of such a virulent race with a genomic clone containing *avr9* resulted in transformants which had become avirulent on tomato genotype *Cf9* (Van den Ackerveken *et al.*, 1992). In addition, disruption of *avr9* in wild type avirulent races of *C. fulvum* resulted in virulence on tomato genotype *Cf9* (Marmeisse *et al.*, 1993). Altogether these results prove that the avirulence gene *avr9* is the only genetic factor responsible for the induction of HR and other defence responses on tomato genotype *Cf9* and that this is mediated by its product, the AVR9 race-specific peptide elicitor.

The AVR9 elicitor is an extracellular peptide, that is produced by *C. fulvum* while growing *in planta*, but not *in vitro*. The *avr9* gene encodes a 63 aa peptide, but *in planta* a 28 aa peptide accumulates predominantly. Expression of *avr9* *in vitro*, as determined by northern blot analysis, has only been detected under conditions of nitrogen starvation. Under these conditions, however, no elicitor activity could be detected in the culture filtrate of *in vitro* grown mycelium (Van den Ackerveken *et al.*, submitted). High level expression of *avr9* *in vitro* would enable us to isolate and purify large quantities of AVR9 elicitor, which can be used in future studies on recognition and signal transduction pathways involved in plant defence. Here, we report on the construction and analysis of transformants of *C. fulvum* producing AVR9 *in vitro* by employing a high expression promoter of *Aspergillus nidulans*. Our experiments provide evidence for the involvement of both fungal and plant proteases in the sequential processing of the 63 aa precursor AVR9 into the mature 28 aa race-specific peptide elicitor.

Results

Isolation of AVR9 producing transformants of C. fulvum

For the development of transformants of *C. fulvum* which constitutively produced high amounts of the AVR9 elicitor *in vitro*, a chimeric gene was constructed (designated pCF22) which consisted of the *A. nidulans gpd* promoter fused to the coding and termination region of the *C. fulvum avr9* gene (Fig. 1). Co-transformation of race 5 of *C. fulvum* with pAN7-1 which contained the hygromycin B resistance marker and pCF22 resulted in stable hygromycin B resistant transformants. Five of these transformants were grown in liquid shake culture and the culture filtrate was assayed for elicitor activity on tomato. The culture filtrate of three of these transformants induced intense necrosis in genotype *Cf9* but not in cultivar MM (lacking resistance gene *Cf9*). Thus, these three transformants produced the AVR9 elicitor *in vitro*. Southern analysis of these AVR9 producing transformants indicated that 5-20 copies of pCF22 were integrated into their genomes.

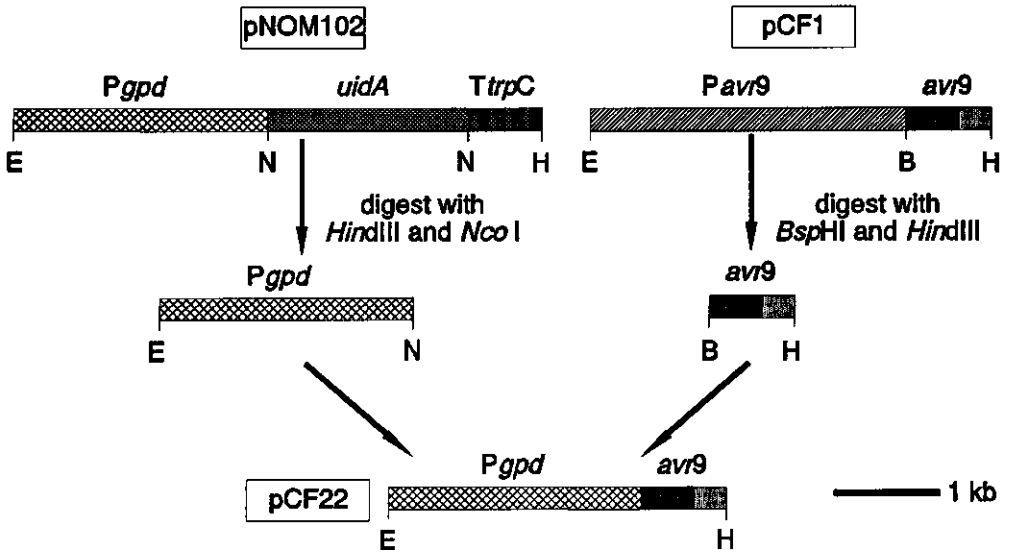

Figure 1. Construction of the P*gpd* - *avr*9 gene fusion. The coding and termination region of the *Cladosporium fulvum avr*9 gene was removed from plasmid pCF1 (Van den Ackerveken *et al.*, 1992) by digestion with *Bsp*HI and *Hin*dIII. The *uidA* gene and *trp*C terminator were removed from plasmid pNOM102 (Roberts *et al.*, 1989) by digestion with *Hin*dIII and *Nco*I and the plasmid containing the high level expression promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (P*gpd*) of *Aspergillus nidulans* (Punt *et al.*, 1988) was isolated. The P*gpd* was subsequently fused to the *avr*9 coding region at the translation start codon present on the *Bsp*HI site, resulting in plasmid pCF21. (*uidA*: ß-glucuronidase coding region, T*trp*C: terminator of *trp*C gene of *A. nidulans*, P*avr*9: promoter of *avr*9 gene of *C. fulvum*, E: *Eco*RI, N: *Nco*I, B: *Bsp*HI and H: *Hin*dIII).

Isolation, purification and characterization of AVR9 from culture filtrate

The AVR9 race-specific elicitor isolated from IF of *C. fulvum*-infected tomato leaves is a 28 aa peptide (Scholtens-Toma and De Wit, 1988) which migrates on low pH PAGE because of its slightly basic nature (Fig. 2, lane 1). Analysis of the culture filtrates of *in vitro*-grown *C. fulvum* transformants producing AVR9 by low pH PAGE showed that the filtrates contained peptides with an electrophoretic mobility different from AVR9 isolated from IF (Fig. 2, lane 2). These same peptides were produced by all three transformants. Although these peptides had a lower mobility on low pH gel, they had similar necrosis-inducing activity on tomato genotype *Cf9* as the 28 aa peptide. Purification of these peptides from culture filtrates by preparative cation exchange chromatography and reversed phase chromatography resulted in an enriched mixture of several peptides with necrosis-inducing activity on tomato genotype *Cf9*. These peptides were subsequently purified by HRLC employing a combination of cation exchange chromatography and reversed phase chromatography. The purified peptides were sequenced from the N-terminus and appeared to be precursors of the 28 aa peptide elicitor. The obtained sequence of 20 N-terminal aa of a mixture of 32 and 33 aa AVR9 peptides overlapped with the sequence of the 28 aa elicitor which has been completely sequenced three times. The 33 and 34 aa peptides (Fig. 2, lane 3 and 4) occur most abundantly, while the 32 aa peptide is present in relatively small quantities, but comigrates with the 33 aa peptide on low pH PAGE. The minor peptide band in lane 2 (indicated with an asterisk) was characterized as a mixture of 32, 33 and 34 aa. Using the Tricine-SDS PAGE system (Schägger and Von Jagow, 1987), which is recommended for the separation of proteins with a MW < 10 kD, the 28 and 32 to 34 aa peptides comigrated and could not be separated. The purification of the 28 aa peptide as described by Scholtens-Toma and De Wit (1988) involved preparative low pH PAGE, which precluded the possible isolation of other peptides such as isolated from culture filtrate as described above. We wanted to know whether these AVR9 precursors were also present in IF of *C. fulvum*-infected tomato leaves. We therefore fractionated and analyzed proteins present in IF by a procedure similar to the one used for the purification of AVR9-peptides from culture filtrate. Following the preparative purification steps of cation exchange and reversed phase chromatography, the fractions containing elicitor activity were

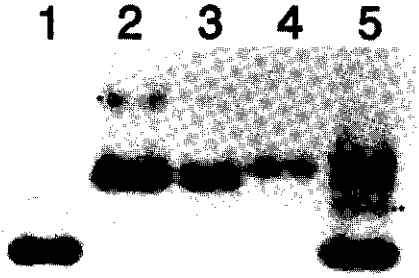

Figure 2. Low pH PAGE profiles of different AVR9 elicitor peptides. The 28 aa peptide, as purified from intercellular fluid of *C. fulvum* (wild type) infected tomato leaves, is a small basic peptide (lane 1). Several different AVR9 peptides are produced *in vitro* by transformants of *C. fulvum* constitutively expressing *avr9* (lane 2); the different peptides (32, 33 and 34 aa) co-migrate and have a reduced mobility as compared to the 28 aa peptide. The two major peptides produced *in vitro* were purified and are the 33 aa (lane 3) and 34 aa (lane 4) AVR9 peptides. The 32, 33 and 34 aa peptides are also present in IF of *C. fulvum* (wild type) infected tomato leaves (lane 5), albeit in significantly lower quantity than the 28 aa peptide. The peptide bands (indicated with asterisks, lane 2 and 5) are less basic forms of the 33-34 and 28 aa peptides, respectively.

Table I. Characteristics of different AVR9 peptides (size, sequence, calculated *Mr* and pI) and the supporting experimental data

Size (aa)	Amino acid sequence	*Mr*	pI	Experimental data
63	MKLSLLSVELALLIATTLPLCWAAALPVGLGVGLDYCNSSCTRAFDCLGQCGRCDFHKLQCVH	6741	7.07	derived from cDNA sequence
40	AALPVGLGVGLDYCNSSCTRAFDCLGQCGRCDFHKLQCVH	4259	7.11	predicted (based on probability)
34	LGVGLDYCNSSCTRAFDCLGQCGRCDFHKLQCVH	3751	7.11	purified and partially sequenced
33	GVGLDYCNSSCTRAFDCLGQCGRCDFHKLQCVH	3637	7.11	purified and partially sequenced
32	VGLDYCNSSCTRAFDCLGQCGRCDFHKLQCVH	3580	7.11	purified and partially sequenced
28	YCNSSCTRAFDCLGQCGRCDFHKLQCVH	3195	7.62	purified and completely sequenced

pooled and analyzed by low pH PAGE. In addition to the major 28 aa peptide the IF contains several other peptides which behave similarly on low pH PAGE and cation exchange HRLC as the AVR9-peptides isolated from culture filtrate (Fig. 2, lane 5). Sequence analysis of the N-terminus of these purified peptides confirmed the presence of 32, 33 and 34 aa AVR9 precursors in IF. The minor peptide band present on the low pH gel, just above the 28 aa elicitor (Fig. 2, lane 5, indicated with a double asterisk) also appeared to be 28 aa in size as determined by N-terminal sequencing. An overview of the different AVR9 peptides characterized so far is presented in Table I.

Proteolytic processing of AVR9 by plant factors

In culture filtrates of *in vitro*-grown *C. fulvum* transformants constitutively expressing the *avr9* gene the 28 aa elicitor could not be detected. The smallest necrosis-inducing peptide isolated from culture filtrate is 32 aa in size. The final processing of AVR9 into the 28 aa peptide, which is the most abundant AVR9 peptide in IF of *C. fulvum*-infected tomato leaves, is therefore thought to take place in the plant. Purified 28 aa and 34 aa peptide elicitors were radioactively labeled with ^{125}I, which is incorporated into the amino acid tyrosine and results in mono- or di-iodotyrosine (Fig. 3B, lane 1 and 2, respectively). Iodinated AVR9 remains biologically active as tested in a pilot experiment using unlabeled NaI (results not shown). The labeled peptides were incubated with IF from healthy tomato plants at 37°C for 2 hours. Analysis of the reaction mixtures by low pH PAGE revealed that factors present in the IF of both tomato cultivar MM and genotype *Cf9* were able to process the 34 aa elicitor into the 28 aa peptide elicitor (Fig. 3A). Proteolytic activity was optimal at acidic pH (2-7) and marginal at alkaline pH (>7) (Fig. 3A, and data not shown). The proteolytic activity in IF was destroyed by boiling IF for 5 minutes prior to incubation (Fig. 3B, lane 6). No reduced proteolytic activity was observed when pepstatin, an inhibitor of acidic aspartyl endoprotease of tomato (Rodrigo *et al.*, 1989), was added to the reaction mixture containing the 34 aa peptide (Fig. 3B, lane 4).

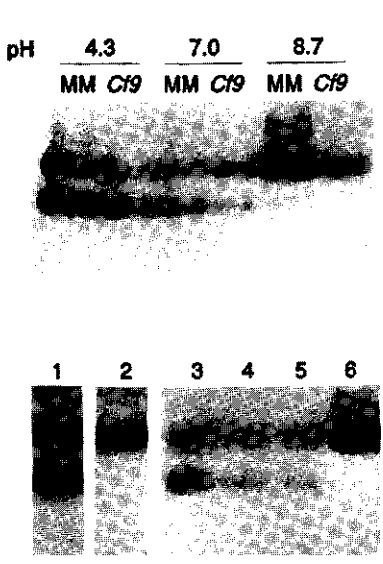

Figure 3. Proteolytic cleavage of the 34 aa AVR9 peptide by plant factors. Autoradiograph of ^{125}I-labeled AVR9 peptides separated by low pH PAGE. **(B)** Untreated 28 aa (lane 1, major lower band) and 34 aa (lane 2) peptides can be separated by low pH PAGE as they significally differ in their pI. **(A)** Labeled 34 aa peptide was incubated with 200 μl of IF from healthy tomato cultivar MM and genotype *Cf9* for 2 hours at 37°C at different pH values. The proteolytic activity is optimal at acidic to neutral pH and marginal at alkaline pH. **(B)** The processing of the 34 aa peptide into the 28 aa form at pH 4 (lane 3) is not inhibited by pepstatin (40 $\mu g.ml^{-1}$, lane 4) nor by ethanol, a solvent for pepstatin (lane 5). Boiling the IF prior to incubation with the 34 aa peptide resulted in loss of proteolytic activity of IF (lane 6).

Discussion

The avirulence gene *avr9* is highly expressed in *C. fulvum* when growing inside tomato leaves (Van Kan *et al.*, 1991), while *C. fulvum* grown *in vitro* does not produce any AVR9 elicitor. High level expression of *avr9 in vitro* has been accomplished by expression of *avr9* under control of the heterologous *gpd* promoter of *A. nidulans*. In *A. nidulans* the *gpd* promoter constitutively expresses the *gpd* gene encoding glyceraldhyde-3-phosphate dehydrogenase (GPD) (Punt *et al.*, 1988). In *Saccharomyces cerevisiae*, GPD may account for 5% of total cellular proteins (Krebs *et al.*, 1953). Transformants of *C. fulvum* containing the P*gpd-avr9* fusion produced and excreted a set of AVR9 race-specific elicitors in culture filtrates of *in vitro*-grown mycelium; these filtrates induce HR in tomato plants carrying the resistance gene *Cf9*.

The AVR9 peptides from culture filtrate were purified to homogeneity, using a combination of cation exchange chromatography and reversed phase chromatography, and sequenced from the N-terminus. Instead of the 28 aa AVR9 peptide, as found in *C. fulvum*-infected tomato leaves, the culture filtrate contained a mixture of 32, 33 and 34 aa peptides. The same peptides could also be detected in IF of *C. fulvum*-infected tomato leaves. These peptides were not isolated before since the purification method described previously (Scholtens-Toma and De Wit, 1988) involved preparative low pH PAGE followed by excision of the band containing the 28 aa peptide and thus precluded the isolation of the 32, 33 and 34 aa peptides. The reduced mobility of the larger forms of AVR9 on low pH PAGE is due to the presence of an additional aspartic acid residue (D) reducing the pI. The peptides (indicated with asterisks, Fig. 2, lane 2 and 5) with reduced mobility on low pH PAGE but with identical N-terminal sequences might be explained by deamination of asparagine or glutamine residues resulting in a less positive charge (Ahern and Klibanov, 1985).

Plant factors mediate the processing of the 34 aa AVR9 peptide, produced by *C. fulvum in vitro*, into the 28 aa form (Fig. 3). Radio-labeled (^{125}I) 34 aa AVR9 elicitor was incubated with IF isolated from healthy plants and subsequently analyzed by low pH PAGE. The processing was inactivated by boiling the IF before incubation with the labeled peptide substrate, suggesting enzymatic cleavage by plant proteases. Alkaline (Vera and Conejero, 1988) and acidic proteinases (Rodrigo *et al.*, 1989) of tomato have been reported to be involved in degradation of plant proteins. The processing of the 34 aa peptide is optimal at acidic pH, suggesting the involvement of an acidic aspartyl proteinase. However, pepstatin, which was reported to inhibit a tomato aspartyl proteinase (Rodrigo *et al.*, 1989) did not inhibit cleavage of the 34 aa peptide. The pH in the apoplast is approximately 5.5, indicating that processing can occur in the tomato leaf.

The results described here allow us to propose a model for the processing of AVR9 (Fig. 4). The precursor protein encoded by the *avr9* gene is 63 aa long as deduced from the cDNA sequence (Van Kan *et al.*, 1991). The mature elicitor peptide, as isolated from *C. fulvum*-infected tomato leaves, consists of the carboxy-terminal 28 aa of the precursor

protein (Scholtens-Toma and De Wit, 1988). The most probable signal sequence cleavage site (Von Heijne, 1986) is located between amino acids Ala (23) and Ala (24) as predicted by the program SigSeq which is included in the Sequence Analysis Software Package of the Genetics Computer Group (Devereux *et al.*, 1984). The resulting extracellular peptide of 40 aa has not been identified experimentally. Extracellular proteases produced by *C. fulvum* are most probably directly processing the 40 aa peptide into the intermediate forms of 32-34 aa. The final step in the processing is mediated by plant factors and results in a stable mature 28 aa race-specific peptide elicitor. We do not know whether the predicted 40 aa peptide can be directly processed into the 28 aa peptide by the plant or whether fungal proteases play an essential role in the formation of 28 aa race-specific peptide elicitor *in vivo*. The specific necrosis-inducing activity of the 32, 33 and 34 aa peptides is indistinguishable from the 28 aa peptide. Whether processing of 32, 33 and 34 aa peptides into the 28 aa form by the plant is essential for elicitor activity is not known.

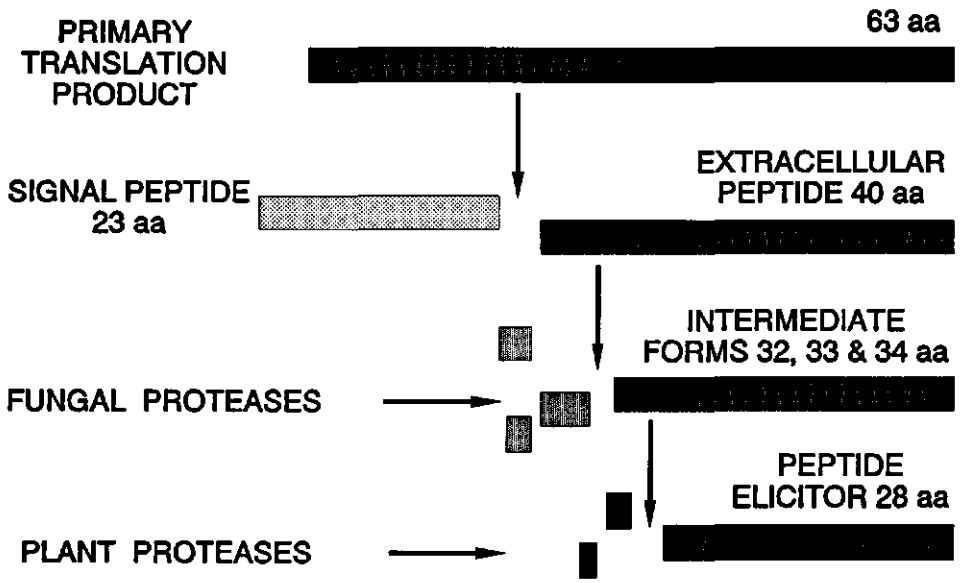

Figure 4. Model for the processing of the AVR9 precursor *in vivo*. The primary *avr9* translation product of 63 aa is excreted by *Cladosporium fulvum* into the intercellular space of tomato leaves during infection, after removal of a 23 aa signal peptide. The 40 aa precursor protein is subsequently cleaved by fungal proteases into 32, 33 and 34 aa peptides. Plant proteases mediate the final cleavage to form the stable 28 aa race-specific peptide elicitor. The model does not exclude the possibility that the excreted 40 aa precursor is directly cleaved by plant proteases to form the mature 28 aa peptide in one single step.

Our results indicate that several processes occur either before or during the recognition of the AVR9 elicitor by tomato genotype *Cf9*. Once the pathogen enters the leaf, the expression of the avirulence gene *avr9* is induced, possibly by the nutritional environment in the leaf or by unknown plant factors (Van den Ackerveken, submitted). The extracellular AVR9 peptide

is instantaneously processed by both fungal and plant proteases. However, we do not know whether the maturation is essential for elicitor activity, as processing of AVR9 peptides occurs too quickly to be studied separately from biological activity of the peptides.

The involvement of both host and pathogen in the formation of elicitors as described here is not unique. In many other host-pathogen interactions non-specific elicitors are formed which are released from cell walls. For example, ß-1,3-glucanases from soybean were shown to release elicitor-active carbohydrates from cell walls of *Phytophthora megasperma* (Keen and Yoshikawa, 1982). In another system, the PGIP (polygalacturonase inhibiting protein) from *Phaseolus vulgaris* inhibits the activity of fungal polygalacturonases, prolonging the life-time of oligogalacturonide elicitors (Cervone *et al.*, 1989). Oligogalacturonides with a degree of polymerization higher than nine act as elicitors of phytoalexin accumulation (Nothnagel *et al.*, 1983). In the absence of PGIP, pectic fragments are quickly degraded by polygalacturonases to monomeric forms which have no elicitor activity.

The ^{125}I-labeled AVR9 peptides are currently used as ligands in receptor binding studies. A hypothetical receptor in the resistant host plant, which might be the primary product of the complementary resistance gene *Cf9*, is proposed to recognize the AVR9 peptide elicitor. Isolation of the *Cf9* gene is a major goal of future studies.

Experimental procedures

Subculture of C. fulvum and AVR9 bioassay

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). AVR9 elicitor activity on tomato (*Lycopersicon esculentum* Mill.) was assayed by injection of 50 µl samples into leaves of cultivar Moneymaker (MM, no *Cf* resistance genes) and a near-isogenic line of MM carrying resistance gene *Cf9* (genotype *Cf9*) (De Wit and Spikman, 1982). One to two days after injection, AVR9 elicitor activity was visible as necrosis (HR) on genotype *Cf9* but not on cultivar MM.

Construction of the avr9 expression vector

The *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter (Punt *et al.*, 1988) was employed to constitutively express the *avr9* gene of *C. fulvum* (Fig. 1). The *avr9* coding and termination region (800 bp) were isolated from plasmid pCF1 (Van den Ackerveken *et al.*, 1992) by digestion with *Bsp*HI and *Hin*dIII. The *uidA* gene and *trp*C terminator were deleted from plasmid pNOM102 (Roberts *et al.*, 1989) by digestion with *Hin*dIII and *Nco*I and replaced by the *avr9* coding and termination region, resulting in plasmid pCF22.

Isolation of AVR9 producing transformants of C. fulvum

After race 5 of *C. fulvum* was grown for 48 hours in liquid B5 medium, the mycelium was harvested and used for the isolation of protoplasts (Harling *et al.*, 1988). Co-transformation (Oliver *et al.*, 1987) of 10^7 protoplasts with 2 μg pAN7-1 containing the hygromycin B resistance gene (*hph*; Punt *et al.*, 1987) and 4 μg pCF22 resulted in stable hygromycin B resistant transformants. Five of these transformants were assayed for the production of AVR9 elicitor *in vitro* and analyzed by Southern blot analyses.

Purification of AVR9 elicitor from culture filtrate

Transformants producing AVR9 elicitor *in vitro* were grown in liquid shake cultures for 7 to 10 days in 50 ml B5-medium in 300 ml conical flasks at 22°C and 100 strokes.min^{-1}. Cell-free culture filtrate was obtained by filtration of the cultures through filter paper over a Büchner funnel. To 500 ml of culture filtrate one volume of acetone was added and the majority of high molecular weight proteins was precipitated overnight at -20°C. After centrifugation, the supernatant was collected and acetone was removed in a rotary evaporator at 50°C. Sodium phosphate buffer (1M, pH 5.5) was added to the remaining supernatant to a final concentration of 20 mM and adjusted to pH 5.5 with H_3PO_4. The conductivity of the sample was adjusted to < 4 mS cm^{-1} by dilution with 20 mM sodium phosphate buffer (pH 5.5). The buffered sample was applied overnight to a CM-Sephadex C-25 column (10 x 2.2 cm), which was pre-equilibrated with 20 mM sodium phosphate buffer, pH 5.5. The column was subsequently washed with 20 mM sodium phosphate buffer, pH 5.5, until the absorbance at 280 nm of the effluent returned to baseline level. AVR9 peptides were eluted by washing the column with 20 mM sodium phosphate buffer, pH 8.0. The fractions containing AVR9 elicitor activity, as assayed by HR induction on genotype *Cf9* of tomato, were pooled and TFA was added to a concentration of 0.1% (v/v). The sample was subsequently applied to a Sep-Pak C18 cartridge (Waters), a reversed phase cartridge for rapid desalting, that had been preconditioned with 10 ml 90% (v/v) acetonitrile, 0.1% (v/v) TFA followed by 10 ml of 0.1% (v/v) TFA. Following application the cartridge was washed with 10 ml 0.1% (v/v) TFA. The elicitor peptides were eluted from the cartridge with 10 ml 90% (v/v) acetonitrile, 0.1% (v/v) TFA. Acetonitrile was removed from the eluate in a rotary evaporator at 50°C and the remaining sample was freeze-dried. High resolution purification was achieved by reversed phase HRLC (High Resolution Liquid Chromatography, BioRad) using a SuperPac Pep-S column (5 μm, C2/C18, 4 x 250 mm, Pharmacia). The sample (200 μl in 0.1% (v/v) TFA) was applied to the column which was pre-equilibrated with 0.1% (v/v) TFA. The column was eluted with a linear gradient of 0.1% (v/v) TFA to 90% (v/v) acetonitrile, 0.1 % (v/v) TFA in 30 min at a flow rate of 1 ml.min^{-1}. Fractions of 0.5 ml were collected and freeze-dried. The dried protein fractions in individual peaks were pooled and dissolved in 20 mM Mes, pH 5.5. The sample (200 μl) was applied to a MA7S cation exchange column (HRLC, 7.8 x 50 mm, BioRad) which was pre-equilibrated with 20 mM Mes, pH 5.5. Following application, the column was eluted with a gradient of 20 mM Mes, pH 5.5 to 20 mM Mes, pH 5.5, 0.4 M NaCl in 30 min at a flow

rate of 1 $ml.min^{-1}$. Individual peaks containing elicitor activity on genotype *Cf9* were analyzed by low pH PAGE.

Polyacrylamide gel electrophoresis (PAGE)

Low pH PAGE was performed on 15% (w/v) polyacrylamide slab gels under non-denaturing conditions using pyronine Y as a front marker (Reisfeld *et al.*, 1962). Following electrophoresis at 200 V, the gels were stained and fixed according to Steck *et al.* (1980). Omitting formaldehyde in the staining and destaining solutions resulted in loss of AVR9 peptides from the gel. PAGE under denaturing conditions was performed on Tricine-SDS-PAGE gels (16.5%T, 6%C) as described by Schägger and Von Jagow (1987).

Peptide sequencing

Purified peptides were sequenced on a gas-phase-sequenator (SON, sequence facility, Leiden, The Netherlands). At least five N-terminal aa were determined for all peptides. Twenty aa were determined for a mixture of 32 and 33 aa peptides. The 28 aa peptide elicitor was completely sequenced.

Radio-Iodination of AVR9 peptides

AVR9 peptides of 28 and 34 aa were radiolabeled by iodination with $Na^{125}I$ (Amersham) using Iodobeads (Pierce) as a catalyst. One Iodobead was washed with PBS before incubation with 19.5 MBq $Na^{125}I$ in 95 μl PBS for 5 minutes in an eppendorf vial. AVR9 peptide was added (5 μl, 5 nmol) and incubated for an additional 15 minutes at room temperature. Following incubation the reaction mixture (100 μl) was transferred to a new vial. The remaining Iodobead was washed with 100 μl PBS. The washing fluid was combined with the reaction mixture (200 μl) and applied to a Sep-Pak C18 cartridge to remove the free ^{125}I from labeled AVR9 elicitor which binds. The labeled peptides were eluted from the column with 90% (v/v) acetonitrile, 0.1% (v/v) TFA. The eluted fractions were freeze-dried and redissolved in milli-Q water. The specific activity of the labeled 34 aa AVR9 was 1 to 2 $MBq.nmol^{-1}$ as determined by liquid scintilation counting.

Proteolytic activity assay

Proteolytic processing by plant factors of AVR9 produced by *C. fulvum in vitro* was studied by using ^{125}I-labeled 34 aa elicitor peptide. Intercellular fluid (200 μl) isolated from healthy tomato plants (De Wit and Spikman, 1982) was incubated with labeled AVR9 elicitor (5 μl, 50,000 cpm) for 2 hours at 37°C. The buffers used were: (i) acidic buffer; 84 mM citric acid, 32 mM Na_2HPO_4, pH 4 (Rodrigo *et al.*, 1989), (ii) alkaline buffer; 150 mM Tris-HCl, 2 mM $CaCl_2$, 0.1 mM DTT, pH 9 (Vera and Conejero, 1988) and (iii) unbuffered; IF adjusted to pH 7 with NaOH. The effect of the protease inhibitor pepstatin was studied by pre-incubating the assay mixture for 30 min at 4°C with inhibitor (40 $\mu g.ml^{-1}$) before adding labeled AVR9. Since pepstatin was dissolved in ethanol, an equal volume of ethanol was added to a control assay, to a final concentration of 2% (v/v). Following incubation of AVR9

elicitor under different conditions, samples were freeze-dried and analyzed by low pH PAGE.

Acknowledgements

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Chapter 6

Nitrogen limitation induces expression of the avirulence gene *avr9* in *Cladosporium fulvum*; a reflection of growth conditions *in planta*?

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submitted to *Molecular and General Genetics*

Summary

The avirulence gene *avr9* of the fungal tomato pathogen *Cladosporium fulvum* encodes a race-specific peptide elicitor that induces the hypersensitive response in tomato plants carrying the complementary resistance gene *Cf9*. The *avr9* gene is not expressed under optimal growth conditions *in vitro*, but is highly expressed when the fungus grows inside the tomato leaf. In this paper we present evidence for the induction of *avr9* gene expression in *C. fulvum* grown *in vitro* under conditions of nitrogen limitation. Only growth medium with very low amounts of nitrogen (nitrate, ammonium, glutamate or glutamine) induced the expression of *avr9*. Limitation of other macro nutrients or the addition of plant factors did not induce the expression of *avr9*. The induced expression of *avr9* is possibly mediated by a positive-acting nitrogen regulatory protein, homologous to the *Neurospora crassa* NIT2 protein, which induces the expression of many genes under conditions of nitrogen limitation. The *avr9* promoter contains several putative NIT2 binding sites. The expression of *avr9* during the infection process was explored cytologically using transformants of *C. fulvum* carrying an *avr9* promoter - ß-glucuronidase reporter gene fusion. The possibility that expression of *avr9* in *C. fulvum* growing *in planta* is caused by nitrogen limitation in the apoplast of the tomato leaf is discussed.

Introduction

The fungal pathogen *Cladosporium fulvum* Cooke is the causal agent of tomato leaf mould. Colonization of susceptible tomato cultivars by *C. fulvum* is characterized by abundant intercellular fungal growth and the absence of active plant defence (De Wit, 1977). Resistant tomato genotypes, however, are able to recognize *C. fulvum* at an early stage of infection, resulting in the induction of the hypersensitive response and other defence responses which restrict growth of the pathogen to the primary infection site (De Wit, 1986). Recognition is mediated via race-specific elicitors, the primary products of avirulence genes of the pathogen, which are recognized by tomato genotypes carrying the complementary resistance genes (De Wit and Spikman, 1982; Scholtens-Toma and De Wit, 1988; Van den Ackerveken *et al.*, 1992).

The avirulence gene *avr9* of *C. fulvum*, the first fungal avirulence gene cloned, is well characterized. Races virulent on tomato genotypes carrying the resistance gene *Cf9* lack the *avr9* gene and thereby avoid recognition (Van Kan *et al.*, 1991). Virulent races transformed with the cloned *avr9* gene are avirulent on tomato genotype *Cf9* (Van den Ackerveken *et al.*, 1992). Disruption of the *avr9* gene in wild-type avirulent races of *C. fulvum* resulted in mutants virulent on tomato genotype *Cf9* (Marmeisse *et al.*, 1993). These results clearly demonstrate the role of the *avr9* gene in the primary interaction of the pathogen with a resistant tomato genotype and the subsequent induction of defence responses. The intrinsic function of the *avr9* gene for *C. fulvum* is still unknown.

The *avr9* gene is highly expressed during growth of *C. fulvum* inside the tomato leaf, while the expression of *avr9* in *C. fulvum* under normal growth conditions *in vitro* is very low (Van Kan *et al.*, 1991). These observations prompted us to study the mechanisms of induction and/or derepression of *avr9*. The expression of *avr9* in *C. fulvum* grown under different conditions *in vitro* was tested by northern blot analysis. The temporal and spatial expression of *avr9* in *C. fulvum* growing *in planta* was studied by using the *Escherichia coli* ß-glucuronidase reporter gene fused to the *avr9* promoter. Nitrogen limitation appeared to induce *avr9* gene expression, which might be a reflection of growth conditions *in planta*.

Results

Accumulation of avr9 mRNA in C. fulvum grown in vitro

The avirulence gene *avr9* is highly expressed when *C. fulvum* grows *in planta* (Van Kan *et al.*, 1991). The level of *avr9* expression during growth *in vitro* in standard B5 medium is low or undetectable. In order to characterize inducing and/or repressing factors or conditions, the effect of plant factors and different growth media on *avr9* mRNA accumulation in *C. fulvum* grown *in vitro* was studied.

Plant factors were added to 48-hours-old *C. fulvum* mycelium grown in liquid B5

medium and after 24 hours their effect on *avr*9 mRNA accumulation was tested by northern blot analysis. Tomato intercellular fluid (De Wit and Spikman, 1982), tomato conditioned medium and homogenized tomato suspension cells (supernatant and pellet; Wolters *et al.*, 1991) were tested for the induction of *avr*9 gene expression. Northern blot analysis of total RNA did not reveal any *avr*9 mRNA accumulation (data not shown) indicating that under the conditions tested, plant factors do not induce the expression of the *avr*9 gene.

The effect of macro-nutrients was tested by growing *C. fulvum in vitro* for two days in B5 medium and subsequent transfer of the mycelium to different test media. After additional incubation for one day, mycelium was collected and the expression of *avr*9 was tested by northern blot analysis. Replacement of sucrose, the carbon source in B5 medium by glucose and fructose did not result in accumulation of *avr*9 mRNA (data not shown). Limitation of the carbon, sulphur and phosphate sources (reduced with a factor 10 and 100 relative to B5 medium) did not result in any induced expression either (data not shown). Only limitation of nitrogen resulted in increased levels of *avr*9 mRNA indicating that *avr*9 gene expression is induced under nitrogen limiting conditions (see next section).

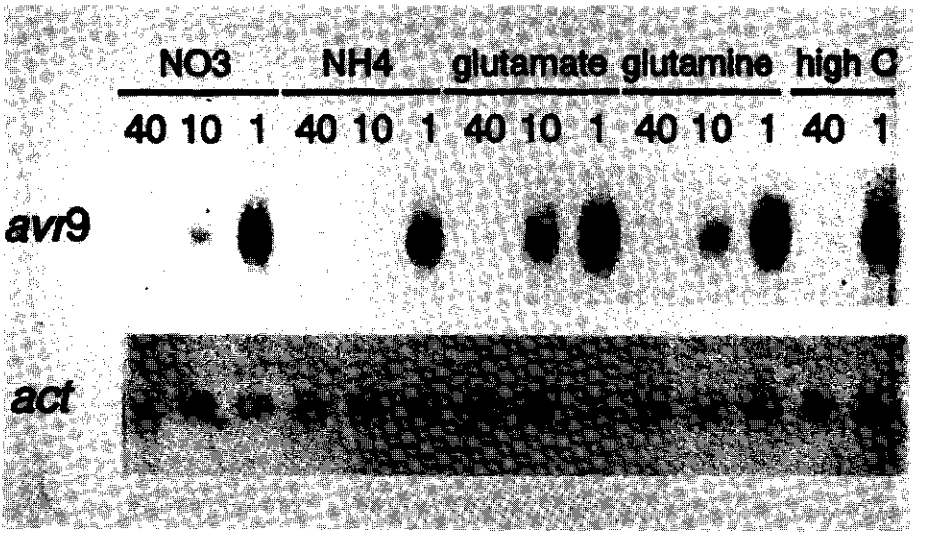

Figure 1. ***avr*9 mRNA accumulation in *C. fulvum* grown *in vitro* in B5 medium containing 40, 10 and 1 mM of four different sources of nitrogen (nitrate, ammonium, glutamate and glutamine) and in B5 medium containing 40 and 1 mM nitrate and 20% sucrose instead of 2% (lanes high C).** Twenty μg of total RNA was electrophoresed in a 1.5% denaturing formaldehyde-agarose gel, blotted onto Hybond N^+ membrane and hybridized with the *avr*9 cDNA and *act* gene (encoding actin, Van den Ackerveken, unpublished) as a constitutive control.

Accumulation of avr9 mRNA under conditions of nitrogen limitation

The effect of nitrogen concentration on *avr*9 gene expression was studied in more detail by testing different sources and concentrations of nitrogen. Figure 1 shows the effect of nitrate,

ammonium, glutamate and glutamine on *avr*9 expression. All these nitrogen sources induced no *avr*9 mRNA accumulation at a concentration of 40 mM. However, high levels of *avr*9 mRNA were observed when nitrogen concentrations were reduced to 1 mM, indicating that the *avr*9 gene expression is strongly induced or derepressed under low nitrogen concentrations. At a concentration of 10 mM, the *avr*9 mRNA level was lower with ammonium compared to the other nitrogen sources. The induced expression of *avr*9 is not caused by a high C/N ratio as medium with a 10 fold higher sucrose concentration did not result in induced *avr*9 expression (Fig. 1). The constitutively expressed *C. fulvum act* gene, encoding actin (Van den Ackerveken, unpublished), was used as internal control probe and demonstrates that comparable amounts of mRNA were loaded on the northern blot.

The effect of nitrate concentration on *avr*9 expression was studied in more detail. Mycelium of *C. fulvum* (grown in B5 medium with 50 mM nitrate for 48 h) was transferred to fresh B5 medium with different concentrations of nitrate (0 - 100 mM) and grown for an additional 24 h. At the end of this growth period the expression of *avr*9 was tested by northern blot analysis. The remaining concentration of nitrate in the culture filtrate after 24 hours of growth was measured, to allow estimation of nitrogen uptake during the growth period. The results are shown in Figure 2. During the 24 h growth period, the concentration of nitrate decreased 7-8 mM. Accumulation of *avr*9 mRNA was detectable when the nitrate concentration at the end of the incubation period was below 1 mM.

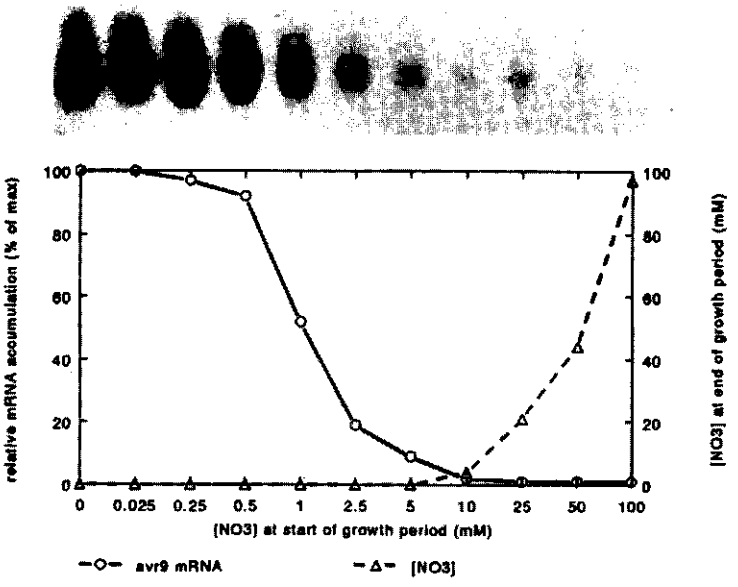

Figure 2. *avr*9 mRNA accumulation (% of maximum) in *C. fulvum* grown in B5 medium with different concentrations of nitrate. The *avr*9 mRNA level was determined by densitometric scanning of a northern blot autoradiograph (shown above graph) containing equal amounts of total RNA hybridized with the *avr*9 cDNA. The nitrate concentration in the culture filtrate after incubation of the fungus for 24 hours was determined and is indicated on the right-hand axis.

Putative nitrogen regulatory elements in the avr9 promoter

Data available on nitrogen regulation in *Neurospora crassa* (Marzluf *et al.*, 1992) and *Aspergillus nidulans* (Caddick, 1992) enabled us to examine the *avr*9 promoter for homologies with known binding sites of regulatory proteins. Six copies of the sequence TAGATA, the consensus sequence for the recognition site of the *N. crassa* NIT2 protein (Fu and Marzluf, 1990), are present in the promoter region -300 to -1 (Fig. 3). Six additonal copies of the core sequence GATA are present in this region as well. *nit-2* is a major positive-acting nitrogen regulatory gene which turns on the expression of many genes under conditions of nitrogen limitation (Fu and Marzluf, 1990).

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      AGGCGCCGTCGCAAACGCTATCGGGTCTTGGATAGGCGGGCAAGATATCTATCGGCTGTT
-300  +---------+---------+---------+---------+---------+---------
      TCCGCGGCAGCGTTTGCGATAGCCCAGAACCTATCCGCCCGTTCTATAGATAGCCGACAA
                                                      1

                                                  2
      GTGCTATTAATATGTATCGCAAGCAGCTAGGTATTAGAAACCTAGATAGCTAGTTGACTT
-240  +---------+---------+---------+---------+---------+---------
      CACGATAATTATACATAGCGTTCGTCGATCCATAATCTTTGGATCTATCGATCAACTGAA

               3
      CATATTGGCTAGATATCTACCTAGAGCAATACAACCTTGAAACAGCTAGGTATAGCAAAC
-180  +---------+---------+---------+---------+---------+---------
      GTATAACCGATCTATAGATGGATCTCGTTATGTTGGAACTTTGTCGATCCATATCGTTTG
                     4

                                        5
      TTCAGTAGCTAGCTAACTTGATATTAACTAGATATCTACCTAGGCAGTAGATCCGGCCGA
-120  +---------+---------+---------+---------+---------+---------
      AAGTCATCGATCGATTGAACTATAATTGATCTATAGATGGATCCGTCATCTAGGCCGGCT
                                          6

                                                                  1
      GAGAGAGATATACAGGTATAAGTAGACAGTAGATCTCTTCTACTCTACTGTTACTAAATC↓
 -60  +---------+---------+---------+---------+---------+---------
      CTCTCTCTATATGTCCATATTCATCTGTCATCTAGAGAAGATGAGATGACAATGATTTAG
```

Figure 3. Putative binding sites in the *avr*9 promoter of *C. fulvum* for a positive-acting nitrogen regulatory protein homologous to the *N. crassa* NIT2 protein (Fu and Marzluf, 1990). The nucleotide sequence of 300 bp of the *avr*9 promoter, upstream of the main transcription start is shown (Van den Ackerveken *et al.*, 1992). The six boxes with the NIT2 consensus sequence (TAGATA) are numbered (1-6). Six additional boxes containing the core sequence (GATA), a putative TATA-box (TATAAGT) and the major transcription start point (position 1, indicated by the arrow) are also indicated.

Studies with the avr9 promoter - GUS fusions

The construction of the *avr*9 promoter - reporter gene GUS fusion is depicted in Figure 4. A perfect fusion of the *avr*9 promoter fragment with the *uidA* start codon was possible because the *avr*9 gene start codon (ATG) was located in the *Bsp*HI restriction site. Three *avr*9 promoter deletions were also constructed. Plasmids pCF23, PCF24 and pCF25 contained fragments of 2, 0.8 and 0.6 kb of the original promoter fused to the *uidA* gene, respectively (Fig. 4).

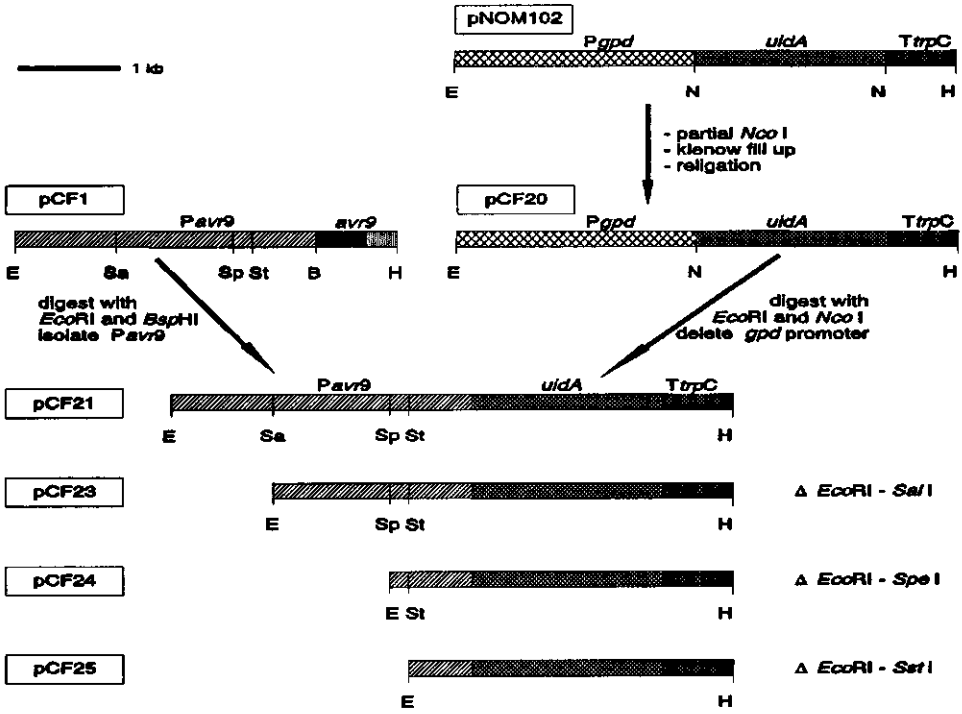

Figure 4. Construction of the *avr*9 promoter - reporter gene GUS fusions.
pNOM102 (Roberts *et al.*, 1989), a plasmid containing the *gpd* promoter of *Aspergillus nidulans* (P*gpd*), the *E. coli uidA* gene, coding for ß-glucuronidase (GUS) and *trp*C terminator (T*trp*C) was modified by removing one *Nco*I site. From the resulting plasmid pCF20 the *gpd* promoter was deleted by digestion with *Eco*RI and *Nco*I. The 3 kb *avr*9 promoter region (P*avr*9) was isolated from pCF1 (Van den Ackerveken *et al.*, 1992) by digestion with *Eco*RI and *Bsp*HI and ligated to the *uidA* gene, resulting in plasmid pCF21. Three promoter deletion derivatives were constructed with 2, 0.8 and 0.6 kb of the original *avr*9 promoter fused to the reporter gene and designated pCF23, pCF24 and pCF25, respectively. B=*Bsp*HI, E=*Eco*RI, H=*Hind*III, N=*Nco*I, Sa=*Sal*I, Sp=*Spe*I and St=*Sst*I.

C. fulvum race 4 and 5 were transformed to hygromycin B resistance with pAN7-1 and co-transforming plasmid (pCF21, 23, 24 or 25). Transformants containing the co-transformed *avr*9-reporter gene GUS fusion were selected by Southern blot analysis. Transformants containing low ß-glucuronidase activity in mycelium grown *in vitro* and high ß-glucuronidase activity in *C. fulvum*-infected tomato leaves were further analysed by northern blot analysis. The *avr*9 and *uidA* mRNA accumulation was determined in *C. fulvum* grown in liquid B5 medium with 50 mM nitrate (low *avr*9 mRNA level) and from *C. fulvum* grown *in planta* (10 days after inoculation, high *avr*9 mRNA level). In the selected transformants, expression of *uidA* (GUS) is detected when the *avr*9 mRNA level is high and not when the *avr*9 mRNA level is low, indicating that the reporter gene *uidA* is regulated similar to the wild type *avr*9 gene (data not shown). The *uidA* mRNA level is much lower than the *avr*9 mRNA level and might be the result of a low *uidA* mRNA stability. The selected transformants, co-regulated for *avr*9 and *uidA*, were used for histochemical analysis of *avr*9 expression. These results and additional data (not shown) obtained by studying these transformants while growing *in planta* indicate that there is no obvious difference between transformants carrying pCF21, -23, -24 and -25, suggesting that full promoter activity is contained within the 0.6 kb promoter fragment present in pCF25.

Localization and timing of expression of avr9 in planta

The different transformants of *C. fulvum* containing *avr*9 promoter - reporter gene GUS fusions showed similar expression patterns during the infection of tomato seedlings. The spatial and temporal expression of *avr*9 was assessed by staining tomato cotyledons infected with several different selected transformants for GUS activity (Fig. 5). Three to four days after inoculation the fungus has penetrated the leaf through the stomata. Six days after inoculation the germinating conidiospores and runner hyphae on the leaf surface showed no significant GUS activity (Fig. 5 **A** & **B**). The penetrating hyphae and intercellular hyphae showed high GUS activity (Fig. 5 **D**, **E** & **F**). Ten days after inoculation the fungus grows abundantly in the intercellular space of the mesophyll. The GUS activity, however, was mainly localized in the mycelium growing near the vascular tissue in the leaf (Fig. 5 **G**) and might be the result of high metabolic activity of the fungus near the vascular tissue.
Twelve to fourteen days after inoculation, a network of hyphae appears in stomatal cavities and conidiophores emerge through the stomata, all showing high GUS activity (data not shown).

Discussion

The specificity of host-pathogen interactions at the molecular level is determined by the interaction between resistance genes of the host and avirulence genes of the pathogen. The tomato-*C. fulvum* interaction provides an excellent model system to study these genes. Two

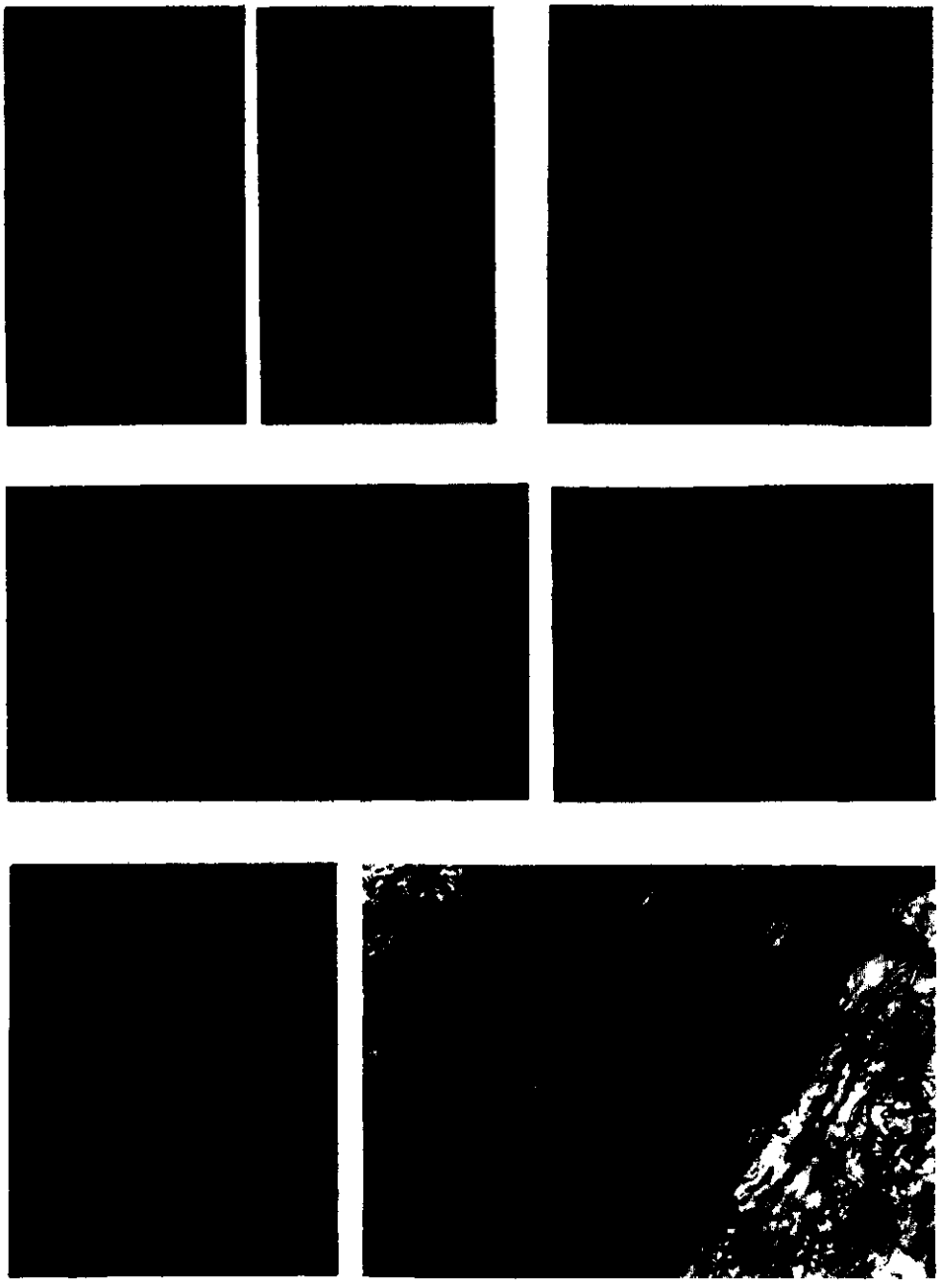

Figure 5. Histochemical localization of GUS activity in cotyledons of the susceptible cultivar Moneymaker inoculated with transformants of *Cladosporium fulvum* containing the *avr*9 promoter - GUS fusion. **A,B)** Conidiospores (C) and runner hyphae (R) on the surface of the ab-axial side of the cotyledon show very low or no GUS activity (6 days post inoculation). **C)** Conidiospores (C) and runner hyphae (R) of a control transformant containing pNOM102 (high constitutive expression promoter, see figure 4) show very high GUS activity (S=Stoma). **D)** The runner hypha (R) from a germinated conidiospore (C) enters the cotyledon via a stoma (S). The penetrating hypha (PH) becomes thickened (H) and shows GUS activity (6 days post inoculation, composite picture taken in different focal planes). **E)** The intercellular hyphae (H) just below the epidermal puzzle cells (P) show very high GUS activity (6 days after inoculation). **F)** The intercellular hyphae (H) continue to colonize the intercellular spaces between the lower mesophyll cells (M). Most hyphae show a high GUS activity, however, some hyphae show very low GUS activity (H →) although they are localized in the same cell layer (8 days post inoculation). **G)** The hyphae (H) near the vascular tissue (V) show high GUS activity, whereas the hyphae (H →) around the mesophyll cells (M) often show low or no GUS activity.

avirulence genes of *C. fulvum*, *avr*9 (Van den Ackerveken *et al.*, 1992) and *avr*4 (Joosten *et al.*, unpublished), have been cloned and were shown to be highly expressed *in planta* compared to normal growth conditions *in vitro* (Van Kan *et al.*, 1991; Joosten *et al.* unpublished). Two putative pathogenicity genes of *C. fulvum*, *ecp*1 and *ecp*2 showed a similar expression pattern (Van den Ackerveken *et al.*, 1993). Unravelling the mechanism of regulation of these genes *in planta* might help to reveal the primary function of these genes for the pathogen.

In this paper we describe the induction of *avr*9 gene expression under conditions of nitrogen limitation. The *C. fulvum avr*4, *ecp*1 and *ecp*2 genes are not induced under these conditions (Van den Ackerveken unpublished). Deprivation of any nitrogen source is sufficient for the induction of *avr*9 expression (Fig. 1). At nitrate concentrations lower than 1 mM the expression of *avr*9 is induced or derepressed (Fig. 2). Under these conditions the *avr*9 gene product could never be detected in the culture filtrate, possibly by the decreased level of protein synthesis under the applied nitrogen limitation conditions.

In *Neurospora crassa* and *Aspergillus nidulans* regulatory genes involved in nitrogen metabolism have been well studied genetically. Nitrogen metabolite repression is mediated by the global regulatory genes *nit-2* and *are*A in *N. crassa* (Marzluf *et al.*, 1992) and *A. nidulans* (Caddick, 1992), respectively. The products of these genes are DNA binding proteins which turn on the expression of many genes under conditions of nitrogen limitation. The *N. crassa* NIT2 protein binds to promoter sequences that contain at least two copies of the sequence TAGATA, or the core sequence GATA (Fu and Marzluf, 1990). The same core sequence is recognized by the mammalian GF1 protein, which has a high homology to the NIT2 and AREA proteins in the zinc finger domain (Marzluf *et al.*, 1992). The NIT2 protein has also been shown to bind to the promoter of the tomato nitrate reductase gene *in vitro* (Jarai *et al.*, 1992) which suggests high conservation of these DNA-binding proteins.

The *avr*9 promoter contains six copies of the TAGATA sequence and an additional set of six copies containing the core sequence GATA (Fig. 3). The induced expression of the *avr*9 gene under conditions of nitrogen limitation could be the result of the binding of a

positive acting regulatory protein homologous to NIT2 and AREA. In future experiments the *C. fulvum avr9* promoter will be tested for AREA regulation in *are*A deletion mutants and constitutive *are*A mutants of *A. nidulans*.

The induced expression of *avr9* under conditions of nitrogen limitation and the putative binding sites in the *avr9* promoter for a major nitrogen regulatory protein suggest that the AVR9 protein might be involved in nitrogen metabolism. Data from *Neurospora* and *Aspergillus* indicate, however, that of the many genes regulated by NIT2 and AREA, some are not directly involved in nitrogen metabolism.

Bacterial plant pathogens possess *hrp* genes, required for the induction of the hypersensitive response in resistant plants and for pathogenicity in susceptible plants. These genes are highly expressed when the bacteria are growing in the host plant, when compared to levels of expression *in vitro*. The *hrp* genes are induced *in vitro* in response to different culture conditions. The *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria* are induced by sucrose and sulphur containing amino acids (Schulte and Bonas, 1992a&b). The expression of the *hrp* genes of *Pseudomonas syringae* pv. *phaseolicola* was shown to be affected by pH, osmotic strength and type of carbon source (Rahme *et al.*, 1992). The avirulence gene *avrB* of *Pseudomonas syringae* pv. *glycinea* could be induced *in vitro* by manipulating the carbon source (Huynh *et al.*, 1989). So far, only the *hrp* loci of *Erwinia amylovora* were clearly shown to be induced under conditions of nitrogen deficiency (Wei *et al.*, 1992).

The bacterial genes described above and the avirulence gene *avr9* of *C. fulvum* are all induced under different growth conditions *in vitro*. Specific plant substances are apparently not required for the expression of these genes. This is in contrast to the nodulation and virulence genes of *Rhizobium* and *Agrobacterium*, respectively (Peters *et al.*, 1986; Stachel *et al.*, 1985), which are induced by specific secondary plant metabolites.

The most interesting question is whether the high level of *avr9* expression in the plant (Fig. 5) is due to low nitrogen concentrations in the apoplast of the tomato leaf where the fungus grows. Nitrogen and other nutrients are taken up from the soil by the plant and may be metabolized in the root before being transported to the leaves through the xylem. The major nitrogen compounds present in the xylem fluid of tomato are glutamine and nitrate (Van Die, 1958). The concentration of nitrogen in the xylem, albeit dependant on the availability and source of nitrogen in the soil, is usually in the range of 0.01% to 0.20% (Pate, 1980). However, the nitrogen content of the apoplast can not be measured accurately. Apoplastic nitrogen concentration is determined by import via the xylem, absorption by cells and export via the phloem (Grignon and Sentenac, 1991). In *C. fulvum*-infected tomato leaves the fungus utilizes this apoplastic nitrogen, thereby reducing the concentration in the apoplast which in turn could induce *avr9* gene expression. The constant flow of nutrients into the apoplast of the leaf via the xylem would support the growth of *C. fulvum* although the absolute nitrogen concentration would remain limiting. Detailed *avr9* promoter analysis will reveal whether the nitrogen responsive elements in the promoter are indeed responsible for the high expression of *avr9* in *C. fulvum* growing *in planta*.

Experimental procedures

Media and growth conditions

Cladosporium fulvum Cooke (syn. *Fulvia fulva* (Cooke) Cif) was grown on potato dextrose agar (PDA) or in liquid B5 medium in shake cultures at 22°C unless otherwise stated (De Wit and Flach, 1979). Ten-day-old PDA cultures of *C. fulvum* were used to prepare spore suspensions for inoculation of plants and for liquid shake cultures (5.10^5 conidia ml^{-1}). Fourteen-day old tomato seedlings were inoculated with spore suspensions as described previously (Van den Ackerveken *et al.*, 1992). Modified B5 medium without nitrogen (B5-N) contained, per liter, the following components: 20 g sucrose, 250 mg $MgSO_4.7H_2O$, 150 mg $NaH_2PO_4.H_2O$, 150 mg $CaCl_2.2H_2O$, 134 mg K_2SO_4, 37.2 mg Na_2EDTA, 27.8 mg $FeSO_4.7H_2O$, 1 mg KI, 1 ml micronutrients; the pH was adjusted to 6.0 with KOH before autoclaving. One ml micronutrient solution contained the following components: 10 mg $MnSO_4.H_2O$, 3 mg H_3BO_3, 2 mg $ZnSO_4.7H_2O$, 0.25 mg $Na_2MoO_4.2H_2O$, 0.025 mg $CuSO_4.5H_2O$ and 0.025 mg $CoCl_2.6H_2O$. Nitrogen was added from a sterile stock solution (1 M nitrogen: 1 M KNO_3, 0.5 M $(NH_4)_2SO_4$, 1 M glutamate or 0.5 M glutamine). The induction experiments *in vitro* were performed with mycelium, which was grown for 48 hours in 25 ml B5 medium containing 50 mM nitrate in 100 ml conical flasks at 22°C and 100 strokes min^{-1}. The mycelium was filtered over miracloth or cheesecloth and rinsed once with the test medium. The collected mycelium was subsequently resuspended in 25 ml test medium and grown for an additional 24 hours under different growth conditions. After 24 hours, the mycelium was collected by filtration over a Büchner funnel, freeze-dried and stored at -20°C until used for RNA analysis.

RNA isolation and northern blot analysis

RNA was isolated from freeze-dried mycelium using guanidine HCl extraction buffer and LiCl precipitation as described by Van Kan *et al.* (1991). Following electrophoresis in denaturing formaldehyde-agarose gels (Maniatis *et al.*, 1982) RNA was blotted overnight to Hybond N^+ membranes with 10xSSC buffer. Northern blots were hybridized overnight with [α-^{32}P]dATP random primed labeled DNA probes at 65°C in modified Church buffer (0.5 M $NaPO_4$, 1 mM EDTA, 7% SDS pH 7.2). After 16 hours hybridization, blots were washed to a final stringency of 0.5xSSC, 0.1% SDS at 65°C. The mRNA accumulation was visualized by autoradiography and quantified by densitometric scanning with a Cybertech CS-1 image documentation and processing system (Cybertech, Berlin).

Determination of nitrate

The concentration of nitrate in culture filtrates was determined essentially as described by Singh (1988). Acetic acid was added to the culture filtrate to a final concentration of 2%. The filtrate was further diluted with 2% acetic acid to give a final absorbance at 540 nm of 0.1 - 1 (equivalent to approximately 0.2 - 2 mM nitrate). Fifty mg of powder mixture (3.7 g citric acid, 0.5 g $MnSO_4.H_2O$, 0.2 g sulphanilamide, 0.1 g N-1-naphtyethelenediamine

dihydrochloride and 0.1 g powdered zinc) was added to each 1 ml sample. The sample was mixed thoroughly and centrifuged for 2 minutes. The absorbance of the cleared supernatant was measured spectrophotometrically at 540 nm and the concentration of nitrate calculated by reference to a standard curve.

Construction of avr9 promoter - GUS fusion

pNOM102, a plasmid containing the *gpd* promoter of *Aspergillus nidulans*, the *E. coli uidA* gene, coding for ß-glucuronidase (GUS, Jefferson *et al.*, 1987) and the terminator of the *A. nidulans trp*C gene (Roberts *et al.*, 1989) was partially digested with *Nco*I. Full-length linearized plasmid DNA was treated with Klenow large fragment DNA polymerase in the presence of dNTPs to fill up the *Nco*I sticky ends. After blunt-end religation and transformation, plasmid pCF20 was selected that contained a single *Nco*I site at the junction between the *gpd* promoter and the *uidA* gene. A 2.5 kb *Eco*RI/*Nco*I fragment containing the *gpd* promoter was deleted from pCF20 and replaced by the *avr*9 promoter present on a 3 kb *Eco*RI/*Bsp*HI fragment that was isolated from pCF1 (Van den Ackerveken *et al.*, 1992), resulting in plasmid pCF21 (Fig. 4). Three *avr*9 promoter deletions (pCF23, -24 and -25) were constructed by digestion of pCF1 (with *Sal*I, *Spe*I or *Sst*I, which are unique in the 3.8 kb *avr*9 insert of pCF1), isolation of the linearized plasmid DNA, creation of an *Eco*RI site by ligation of an *Eco*RI linker, and ligation of the new *Eco*RI/*Bsp*HI fragment in *Eco*RI/*Nco*I digested pCF20 (Fig. 4).

Analysis of C. fulvum transformants

C. fulvum (race 4 and 5) was grown for 48 h in liquid B5 medium, the mycelium was collected and used for the isolation of protoplasts (Harling *et al.*, 1988). Co-transformation (Oliver *et al.*, 1987) of 10^7 protoplasts with 2 μg pAN7-1 containing the hygromycin B resistance gene (*hph*, Punt *et al.*, 1987) and 4 μg of either pCF21, -23, -24 or -25, resulted in stable hygromycin B resistant transformants. Transformants were analysed by Southern blot analysis for the presence and copy number of the co-transformed *avr*9 promoter-GUS constructs. Transformants with a low copy number were subsequently tested by northern blot analysis for the accumulation of *avr*9 and *uidA* mRNA. The expression of both genes was tested in *C. fulvum* growing *in planta* at ten days after inoculation and in *C. fulvum* grown *in vitro* after transfer of 48 h old mycelium to B5 medium with 50 mM nitrate and growth for one additional day.

ß-Glucuronidase assay

Histochemical localization of *avr*9-GUS expression in *C. fulvum* growing *in planta* was assayed by vacuum infiltration of infected tomato cotyledons with a solution consisting of 50 mM sodium phosphate (pH 7.0), 1 mM potassium ferri/ferro cyanide, 0.05% Triton X-100 and 0.5 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl ß-D-glucuronide, Sigma). The infiltrated cotyledons were incubated at 37°C for eight hours in the dark. Finally, cotyledons were

cleared of chlorophyll by incubation in 70% ethanol and analysed by light microscopy.

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Chapter 7

Isolation and characterization of two putative pathogenicity genes of *Cladosporium fulvum*

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adapted from *Molecular Plant-Microbe Interactions 6 (1993), 210-215*

Summary

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 (*ecp*1 and *ecp*2). The DNA-sequences of these *ecp*-genes (encoding extracellular proteins) do not share homology with 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.

Introduction

The interaction between the biotrophic fungal pathogen *Cladosporium fulvum* and tomato 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 defence responses, such as the accumulation of PR proteins, are activated much later as compared to incompatible interactions (Joosten and De Wit, 1989). The accumulation of 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 kD) 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 to P1; Joosten and De Wit, 1988) and ECP2 (Wubben *et al.*, in preparation) 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 *ecp*1 and *ecp*2. 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, a clear hybridization signal was detected specifically in RNA isolated from compatible *C. fulvum*-tomato interactions (results not shown). In order to obtain a 100% matching probe, RNA from a compatible *C. fulvum*-tomato interaction was used as template for RNA-sequencing using the oligonucleotide probe as primer. The sequence of the RNA confirmed the N-terminal amino acid sequence of ECP1, and allowed the synthesis of a perfectly matching oligonucleotide probe (amino acids 11-20, Fig. 1), which was used to screen a λZAP library containing cDNA obtained from *C. fulvum* infected tomato leaves. Several positive clones were selected of which two were analyzed in detail. Both clones contained an insert encoding a protein which corresponded to the amino acid sequence obtained for ECP1. The cDNA insert was subsequently used to screen a genomic library of *C. fulvum*. A 7 kb *Xho*I fragment containing the *ecp*1 gene was subcloned from a purified positive phage (pCF140).

Isolation of the ecp2 gene

Antiserum raised against the ECP2 protein was used to screen a λgt11 expression library containing cDNA obtained from *C. fulvum*-infected tomato leaves. Two positive phages, containing inserts of 600 and 650 bp, were purified and tested on a northern blot containing RNA from *C. fulvum* grown *in vitro*, healthy tomato and *C. fulvum* infected tomato, respectively. The 600 bp *Eco*RI insert was shown to be of fungal origin and hybridized to mRNA which was highly abundant in compatible *C. fulvum* - tomato interactions (results not shown). The cDNA insert was subsequently used to screen a genomic library of *C. fulvum*. Several positive clones were obtained and a 4.3 kb *Eco*RI/*Bam*HI fragment, containing the *ecp*2 gene, was subcloned (pCF170).

Sequence analysis of ecp1 and ecp2

Detailed restriction maps of the subcloned DNA fragments containing the *ecp*1 and *ecp*2 genes were obtained by Southern analysis (Fig. 2). The *ecp*-genes are single copy genes in all races of *C. fulvum* tested (data not shown). The DNA sequence of the coding region and approximately 1 kb of the upstream region was obtained for both genes (Figs. 3 and 4). Introns were identified by comparison of cDNA sequences with genomic sequences. Two introns were found in *ecp*1, and one in the *ecp*2 gene. The transcription start sites, as determined by primer extension, and polyadenylation sites are indicated in figures 3 and 4 for *ecp*1 and *ecp*2, respectively. A possible TATA-box and CAAT-box are present in both genes in the 5'-upstream region.

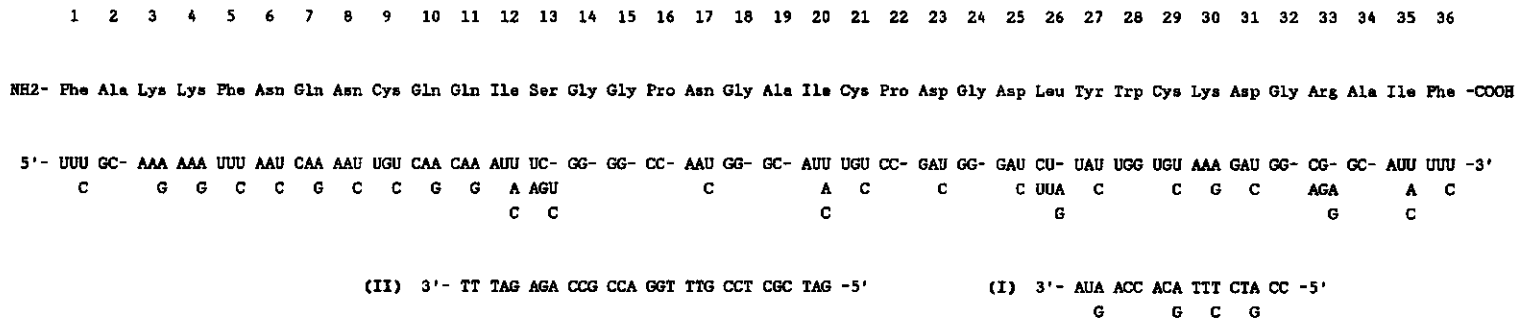

Figure 1. N-terminal amino acid sequence of the purified protein ECP1 with the derived mRNA sequence (-: A, C, G or U). The 17-mer oligonucleotide probe (I) was initially chosen for its low level of degeneracy. The 29-mer oligonucleotide probe (II) was synthesized using mRNA sequence data obtained with the 17-mer probe as primer.

The amino acid sequence which was determined for the purified proteins (ECP1: Fig. 1, ECP2: see below) was confirmed by the cDNA and genomic sequences. The mature protein, as isolated from *C. fulvum*-infected tomato plants, is indicated in bold (Figs. 3 and 4). Both precursor proteins contain a signal sequence which is hydrophobic. The signal peptide cleavage site, as predicted by the computer program SigSeq (GCG), is indicated by the arrows (Von Heijne, 1986). Comparison of both DNA and protein sequences with the GenBank, EMBL, PIR and SwissProt databases (Devereux *et al.*, 1984) did not reveal significant homology to any known sequence.

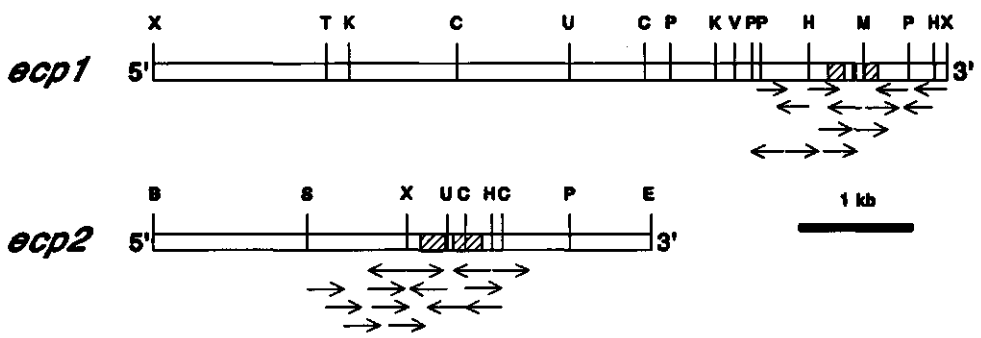

Figure 2. Restriction map of sequences surrounding the *ecp*1 and *ecp*2 open reading frames (ORF, hatched bar). The restriction map is derived from Southern blot analysis of genomic DNA digested with several restriction enzymes and probed with the *ecp*1 and *ecp*2 cDNAs, respectively. The sequencing strategy is indicated by the arrows. B, *Bam*HI; C, *Hinc*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; M, *Sma*I; P, *Pst*I; S, *Sal*I; T, *Sst*I; U, *Pvu*II; V, *Eco*RV; X, *Xho*I

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CTGCAGTGTGCCATGCGCTTATTACAGACGACTTCTCGGGGATACCTGCAGGAAGTGGAC    -605
GTTCGTGCTTTGATGAGTTGAAGCACTCCCCGAGAGTCGAGACCGATTGTGACCGTCATT    -545
TGCGCTTCCACGTTCGCGGTTCGCCGCATTCGAGGCCGGGATGGGTTTGGAAAGTCGCTG    -485
TTAAAGAAAGGCGTAGTTGCCATTCCTGGGCGTCACTTCGGGGCCGCGCGCGATTTAGCT    -425
CATCGAGCCAGGTTGCCACATGCCTGCCCTCCCACGTGCAACGATCATGTTGGTACGAAG    -365
CCGCAGAACACTGCACTTTCACGGACTCGTAACTCGTAACATCTACCAAGGTCATCTCGA    -305
ATCTCGGCCATCTCGCAAGCGCACGGGTATGTCCTCTCACGACCAGGATAAGTTCGCAGT    -245
CCGGACGGCGTATTTACCAGCAAGCTTACGAGATGGCCGCGATTCGAGATGACCCTGGTA    -185
GATGTTATACAATGAGTTGCGGAACTTCGCGGAGCTTCGTAATATTGTGCAGCGTACTGT    -125
ACAATGCTTGATGTAAGCTTGGCACAACATCCAAGAAGCCTGATCGGTGCGGCCAGTGCT    - 65
TGTAGACGGCACCCTAGGTGTGCAACCTATAAAGCAGCCCGTCTGCGGCTGCGCATTATTG    -  5
                                                                  1
CTTCATCACATCCTTAGTTACCGAGACATCACCACTCCCACTTCCCTCCAAGATGCACTT     56
                                                   M  H  F

CGTCACATCTCTCATCGCCGGCGTCGCCATGCTCGCCACGGCGAGCGCCACTGTCCAAGG    116
 V  T  S  L  I  A  G  V  A  M  L  A  T  A  S  A  T  V  Q  G
 ↓
CGGCGCCCCCGTGGACGATCTCAAGTTCGCAAAAAAGTTCAACCAGAACTGCCAGCAAAT    176
 G  A  P  V  D  D  L  K  F  A  K  K  F  N  Q  N  C  Q  Q  I

CTCTGGCGGTCCAAACGGGTAAGACCCTCGACCCTCATCCAACCCACTCGGCCACAATAG    236
 S  G  G  P  N  G ----------------------------------------

ATCGCGAAGACTCCACGAAACTAACACGAATGATACCATCTTAGAGCGATCTGCCCAGAC    296
---------------------------------------- A  I  C  P  D

GGCGACTTGGTATGACCACTCGTACCCCTCAGCGATCGATGCTGTCCTCGAACTAATGCG    356
G  D  L ------------------------------------------------

GGAATGCCCCGGGAACAGTATTGGTGCAAAGACGGTAGGGCCATATTTTGCCAGACATGC    416
----------------Y  W  C  K  D  G  R  A  I  F  C  Q  T  C

CAAACTGGATGTACCGCCGATGAAAATGGCAAGGTCGGCTACTGCAACGAAGGGCCTACA    476
Q  T  G  C  T  A  D  E  N  G  K  V  G  Y  C  N  E  G  P  T

AACCCCAAGTGCCTTTAATGGAGGAAGCATCGCGGAGGCTGAATGCCACCGTTACCATTT    536
N  P  K  C  L
                                                  ▼
GAGACGATGTGGAGAATGTGCCCTTCTACGTCGAGGGGCAGAGCGACCGTACGCCTTACG    596

TAGACATCCAAGCAACGAGGAGACGTTAAGCCCTGTAATTGCTCAAATCGTCTATCCCTC    656
                 ▼
TAGATGCCCAGCTTCCCTTGCCTAAAGCGAAACGTGCGCATCCTGTGAGCGCAACGACTC    716

CAGCTCGGCCTGCAGTGGACCGCTCGTCGATGTATTGGCAGCCACAGCTTCCCGCCCAT    776

CCTCTACACCAAAGCAGACCGGTGTTATACAGCCACACTAGCCCAGTACGGCCCCATACA    836

GCGAGATCGTCGCACTAAACATTAGCGAAGAGCATGATCCACGGCCTACATGTCTTTCGC    896

CCTGTAGTAGACAAACCGCAGACTTCTACATTTATTGAGCGCAACGACCGACGAGCGCTA    956

GCCACACCGTAGCGACGTATAAGCTT  982
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Figure 3. Sequence of the *C. fulvum* gene *ecp*1 (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 (▿).

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GATCGGAGGTGAACAGAGACTGTAGGTGTTTATGAGACGGGGGCAATCCTAGTGCGAGGC   -630
TGTGGTTAGCCGAAAATCGTTGTCAATCGTCTGTCAAATTAGCATCGTCAAGGGTTGCAG   -570
CCAAGGTTTCCTTGTCTTCAGATGGAAACTAGCGGGACTATTTGGCGGTGAGTCTTCAGA   -510
GGGATGCAAGTGGATGCTCGTACTCCCAGGTCGTGACGCGGGCACGTGGCGCTACTCAGAA   -450
CAACGAGTGTCACCACCTTTAGCAACATACAGCGTCTGTCAGCACGGAGAACATCTTTAC   -390
TTAGCGTTCGCAGTGTTTGGCGCCGGCCGATGCTATGATCCTGCTTTCCGCTGGGATAAGA   -330
CGATCTGCACTAACGGGAAATGTAACTCCGCTGCTGACAGACGCTGTACGATTCCAAGAC   -270
GAAGGAGTTTACCGTCTAGGTGTCAAATCCTAGATCCCAGGAAATCCAAATCTGGACCCT   -210
TGGCTACATGAGTTACGAAGCAGGTTGCAACAGTGCACTGCCCAAGTCGAACGCTATGGC   -150
CTGGGTAGGGCGTTTCGTGCGATGCCCAGTCCAAGGATCGTGTCTCAAGATGCAGCCTTA   - 90
CAGTTCGATCAAATTCGCCACCAGAAATGACCTCGAGTGTTGGGAACGGTATTTATAGTC   - 30
                                ▼
CCCTAAGTATGCGCCTCAACGCCCACCAAGTTTACCTTCACCGAACTTCGAACAACAGTA     31
CACTCTCCACACAACACCACTCTTCCTCCTGTGCCAACATCACACGACCATACAACCAGC     91
TTCACGATGCTCTTCAACGCCGCCGCCGCCGCCGTGTTCGCCCCTCTCCTAGTCATGGGC    151
      M  L  F  N  A  A  A  A  A  V  F  A  P  L  L  V  M  G
                ↓
AACGTTCTGCCTCGGAACGCTGGCAACTCGCCCGGCTCAAACGCGCTGCGATGCCTCAACT    211
N  V  L  P  R  N  A  G  N  S  P  G  S  N  R  C  D  A  S  T

TTTAACAACGGCCAAGACTTTGACATTCCACAGGCACCAGTCAATGACTGCCGGCAGATG    271
F  N  N  G  Q  D  F  D  I  P  Q  A  P  V  N  D  C  R  Q  M

GTTGAAAACATCAATAGAGATTCTCAATTCTCAGTGTCCCACAGCTGGGCGAGACCATTC    331
V  E  N  I  N  R  D  S  Q  F  S  V  S  H  S  W  A  R  P  F

GGCGGATATGGCGATTGTACGTCACAGAGCCTCCCACGACAATTCTTGAATATACTGACA    391
G  G  Y  G  D  C----------------------------------------------

TACCATTTCCAGGTGCATTCAACGTCCGCGTCATTGCTGGCTGGCGGAATGGCTTGGTTG    451
-----------  A  F  N  V  R  V  I  A  G  W  R  N  G  L  V  G

GCGGTGCGGATGCCGTTGACCTTCTCACTGATTCTGTCAAAAACTTCGGTGAGGCCAACA    511
   G  A  D  A  V  D  L  L  T  D  S  V  K  N  F  G  E  A  N  K

AGGTTTCCAGCAAGGGCACCTACAACCAAATTGTCTCCGCGGAGGGCGAAGTTACGTGCG    571
   V  S  S  K  G  T  Y  N  Q  I  V  S  A  E  G  E  V  T  C  D

ATTCTGTGGATCGTGGTGGTCAGGTCAGAGTTCAGTGGATTGTTGCCAGCTCATCGTACA    631
   S  V  D  R  G  G  Q  V  R  V  Q  W  I  V  A  S  S  S  Y  N

ACCCGTCCAACGATGACTAGAGGCCGCGTCAGACGGGTTTGCTAGAACCAGGTTGTAGCA    691
   P  S  N  D  D

GAGCTGAACATGGCATTTCTGGTGTCGAAGCTTGGGCATAATCAAATATACAGAGCAACG    751
                ▽
GAATGGATCTTGAAGCTAGTAGTCAACCATACGACTTGATCTTTCAACCACACACAGCTC    811

TGATACAGACATCACAGTTCGGCATCCACACTAAAGCCTTTCGATAACCGTCCATGCCTC    871

CAGCCTGATTCGCAGAATCGTCGAACCAGACCATCATCTCCTGGTCTGCTTCGGCAGCAG    931

TCATGATC  939
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Figure 4. Sequence of the *C. 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 (▽).

Expression of ecp1 and ecp2 in vitro and in planta

The cDNAs of both *ecp*1 and *ecp*2 were used as a probe to hybridize to northern blots containing poly(A)$^+$RNA isolated from an incompatible and a compatible *C. fulvum* - tomato interaction at different times after inoculation (Fig. 5). No hybridization was detected in healthy plants nor in incompatible interactions. The *ecp*2 transcript is present at a low level in *in vitro*-grown *C. fulvum*, whereas the *ecp*1 transcript could not be detected under these conditions. The time course of accumulation of the *ecp*1 and *ecp*2 transcripts is rather 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 (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*.

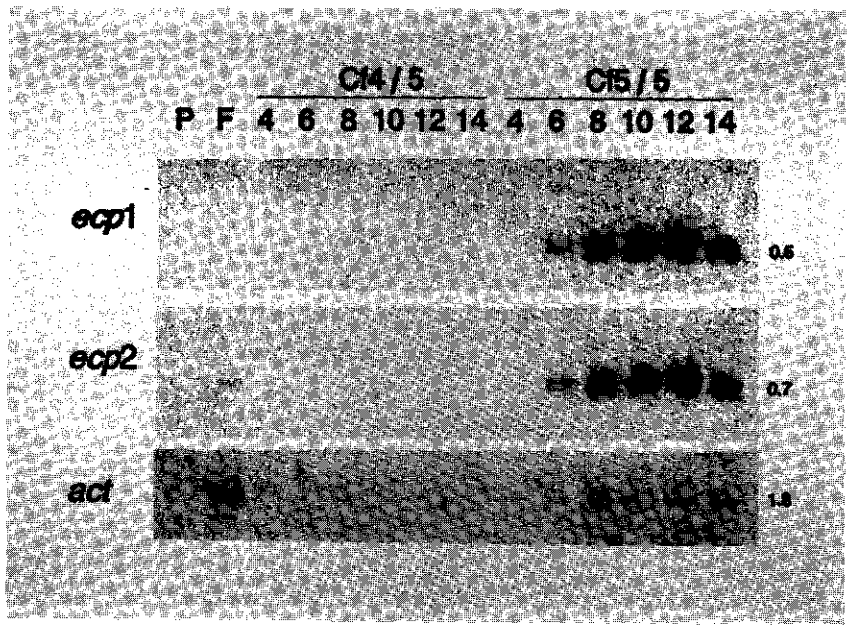

Figure 5. Time course of accumulation of the *ecp*1, *ecp*2 and *act* mRNA. The cDNA-inserts (*ecp*-genes) and genomic subclone (*act*) were labeled by random priming and hybridized to a northern blot containing equal amounts of poly(A)$^+$-RNA isolated from uninoculated tomato genotype Cf5 (lane P), 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, 14 days, respectively) after inoculation. The sizes of the hybridizing mRNAs in kilobases is indicated.

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 kD. Acrylamide gels of 16.5% T and 6% C enabled us to clearly separate proteins in the 5 - 20 kD range. The molecular weight of the ECP1 protein was estimated to be 9 kD, which is in agreement with the molecular weight deduced from the DNA sequence.

To confirm that the cloned *ecp*2 gene indeed encodes the ECP2 protein we purified and sequenced the N-terminal part of the protein. The sequence obtained (NH_2 - 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

Apoplastic fluid of *C. fulvum*-infected tomato leaves contains several low molecular weight proteins (<20 kD) 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 to P1; Joosten and De Wit, 1988) and ECP2 (Wubben *et al.*, in preparation) were purified. Amino acid sequence data of the purified ECP1 enabled us to employ oligonucleotide probes to isolate the corresponding cDNA and gene (*ecp*1). 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 (*ecp*2).

The *ecp*1 gene encodes a precursor protein of 96 amino acids. The processed protein is 65 amino acids in size (7 kD), 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 kD (Joosten and De Wit, 1988), which is much higher than the deduced molecular weight of 7 kD. By using the Tricine-SDS-PAGE system, the molecular weight was estimated to be 9 kD, which is more in agreement with the deduced size of 7 kD. The processing of the 96 amino acids precursor protein to the 65 amino acids mature protein most probably involves two steps. Firstly, 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 *avr*9 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 (Van den Ackerveken *et al.*, 1993).

The processing of the ECP2 protein is similar to that of the ECP1 protein. The *ecp*2 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 acid pre-protein 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 kD from SDS-PAGE (Wubben *et al.*, in preparation) is in good agreement with the 142 amino acids protein as deduced from the DNA-sequence.

The structure and organization of the two cloned *ecp*-genes of *C. fulvum* is typical for filamentous fungi (Figs. 3 and 4). Motifs which 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.*, 1987). 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 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 *ecp*2-gene is located on the CAAG-sequence which is a common transcription initiation site 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.*, 1987).

The *ecp*-genes and the previously cloned avirulence gene *avr*9 (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 *ecp*1 and *ecp*2 (data not shown). Future research using promoter-reporter gene fusions will enable us to define the promoter regions which 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 Wubben *et al.* (in preparation) 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*.

Experimental procedures

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 $5x10^6$ 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 two and three weeks after inoculation (De Wit, 1977; Lazarovits and Higgins, 1976a).

Purification of compatible interaction specific proteins

Intercellular fluid was isolated from the compatible tomato genotype *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 (Wubben *et al.*, in preparation), 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 genotype *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-^{32}P]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 genotype *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 and Schüell), soaked in 0.25% IPTG and dried, were placed on the top agar and incubated for 4 hours 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% BSA and 0.02% NaN_3). The antigen-antibody complexes were detected with the BioRad Immun-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 [α-^{32}P]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 described (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 act*A 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).

Acknowledgements

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Chapter 8

General Discussion

In the last decade significant progress has been made in unraveling host-pathogen interactions at the molecular level. Avirulence genes of many bacterial and a few fungal plant pathogens have been cloned and characterized in detail. The gene-for-gene hypothesis as proposed more than 50 years ago by Flor (1942) is still standing and is now supported by molecular data. Aspects of pathogenicity can now be analysed by employing molecular genetic techniques to provide conclusive evidence on the role of putative pathogenicity genes in host-pathogen interactions. In this chapter major advances in the molecular understanding of avirulence and pathogenicity of bacterial and fungal plant pathogens, with emphasis on *Cladosporium fulvum*, are discussed.

The biological data on the *C. fulvum* - tomato interaction presented in the preceding chapters can be integrated in two conceptual models. Pathogenicity factors of *C. fulvum* and their possible role in establishing basic compatibility on tomato are described in the first model. The second model describes race-specific incompatibility as a result of early recognition of avirulent races of *C. fulvum* by resistant tomato cultivars, carrying the complementary genes for resistance.

Basic compatibility

The ability of a pathogen to successfully infect and colonize a particular host plant is called basic compatibility (Ellingboe, 1976; Heath, 1981). Most pathogens are able to colonize only a limited number of host plants. Successfull infection of a host requires a range of pathogenicity factors, among which are attachment to the host plant (Hamer *et al.*, 1988), formation of penetration structures (Staples *et al.*, 1986), degradation of host cuticle and cell walls (Collmer, 1986; Dickman *et al.*, 1989), toxin production (Panaccione *et al.*, 1992), phytoalexin detoxification (Van Etten *et al.*, 1989) and suppression of defence responses (Heath, 1982), etc.

Aspects of pathogenicity and basic compatibility have been studied in bacterial plant pathogens by using non-pathogenic mutants. Genes involved in pathogenicity have been cloned by complementing these mutants with a genomic library of wild-type pathogens. In this way the so-called *hrp* genes have been cloned from several bacterial plant pathogens (Willis *et al.*, 1991). The *hrp* genes are involved in pathogenicity on a susceptible host as well as in the induction of the hypersensitive response on resistant hosts. Recently, homology between *hrp* gene products of bacterial plant pathogens and proteins of mammalian bacterial pathogens involved in excretion of pathogenicity factors has been observed (Gough *et al.*, 1992).

All pathogenicity genes from plant pathogenic fungi characterized so far have been cloned by function, such as cutinase (Dickman *et al.*, 1989), pisatin demethylase (Weltring *et al.*, 1988) and toxin synthetase (Panaccione *et al.*, 1992). Pathogenicity genes with as yet unknown functions are difficult to isolate unless other approaches are addressed. Inventarization and analysis of fungal genes expressed during pathogenesis might provide a broader view on the molecular basis of pathogenicity. One such approach is the differential hybridization of a genomic library of the fungus with cDNA synthesized on mRNA from *in vitro* grown and *in planta* grown fungus. Nine *in planta* induced genes of *Phytophthora infestans* were isolated in this way (Pieterse *et al.*, 1993). In the *C. fulvum* - tomato interaction, *in planta* induced fungal genes have been characterized and cloned via their protein products which specifically accumulate in infected tomato leaves but not in the fungus grown *in vitro* (Chapter 7).

The knowledge on factors of *C. fulvum* involved in establishing and maintaining basic compatibility on tomato is very limited. A number of putative pathogenicity factors of *C. fulvum* is proposed and depicted in the first model describing the infection of a susceptible tomato genotype by virulent races of *C. fulvum* (Fig. 1). These races are not recognized by resistant tomato genotypes as they either do not produce a race-specific elicitor or the produced elicitor is not recognized. Besides, in susceptible tomato genotypes elicitors cannot be recognized due to the absence of the corresponding resistance gene (see next section). *C. fulvum* can grow abundantly in the leaves of susceptible plants while no defence responses are induced. Possibly suppressors produced by *C. fulvum* abolish the induction of non-

specific plant defence responses. Also, host factors could suppress the activity of non-specific glycoprotein elicitors produced by the fungus (Peever and Higgins, 1989). Therefore it might be concluded that *C. fulvum* is adapted so well to its host plant tomato that none of its excreted compounds induce non-specific defence responses while the fungus grows inside the leaf.

C. fulvum has to obtain all nutrients necessary for growth and reproduction from the intercellular spaces between the mesophyll cells. Possibly, pathogenicity factors interfere with metabolism and translocation of nutrients in the host plant. Invertases, for instance, excreted by *C. fulvum* in the apoplast convert sucrose into glucose and fructose and might thereby interfere with sugar transport in the host (Joosten *et al.*, 1990). It has been shown that yeast invertase targeted to the apoplast in tomato, tobacco and *Arabidopsis* leaves significantly reduces the source activity of photosynthesizing leaves (Von Schaewen *et al.*, 1990; Dickinson *et al.*, 1991). It was suggested that phloem loading from the apoplast is achieved by sucrose carriers and that glucose and fructose, the products of invertase activity, can no longer be loaded effectively. Similarly, *C. fulvum* could reduce the phloem loading in tomato by its invertase activity, preventing translocation of carbon sources from the infected leaf. Possibly, the conversion of monosaccharides to mannitol enhances this effect, as mannitol cannot be utilized by the host (Joosten *et al.*, 1990).

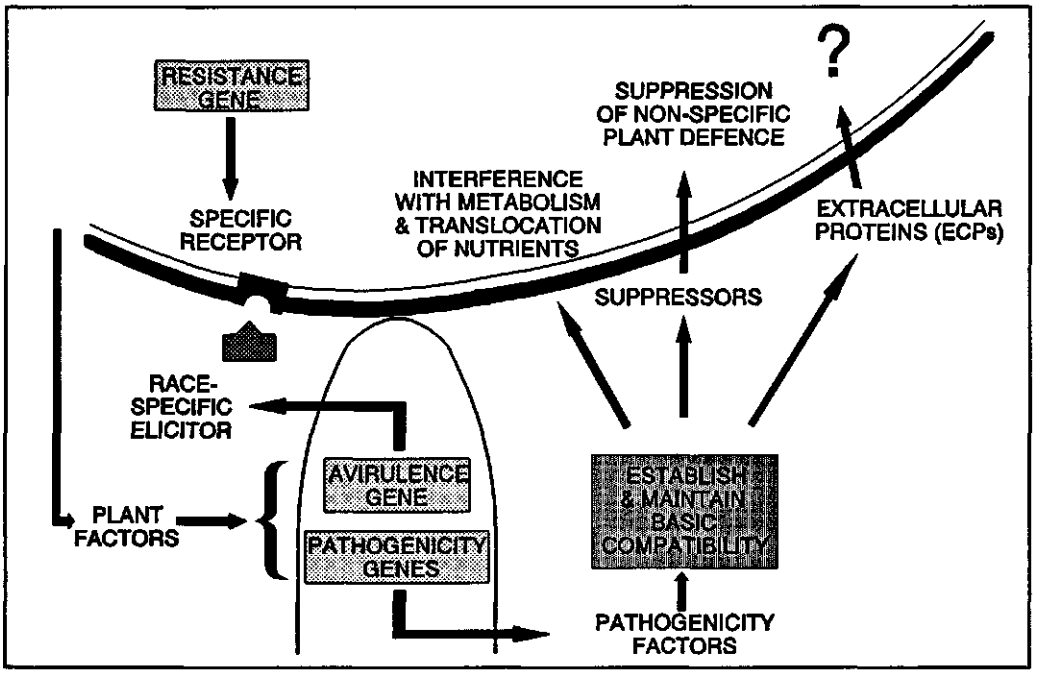

Figure 1. Schematic representation of events involved in the establishment of basic compatibility between virulent races of *C. fulvum* and susceptible tomato cultivars (details discussed in text; adapted from Van den Ackerveken and De Wit, 1993).

In planta induced genes of *C. fulvum*, such as the putative pathogenicity genes *ecp*1 and *ecp*2 (chapter 7) and the avirulence genes *avr*9 (chapter 2) and *avr*4 (Joosten *et al.*, in preparation), represent a group of genes with no known primary function for the pathogen. Their possible involvement in pathogenicity can now be addressed by gene disruption (Chapter 4). Studies on the regulation of expression of these genes and the possible involvement of plant factors in the induction gene expression might provide more information on the function of these genes for the pathogen. Future research will likely reveal thus far unknown strategies employed by *C. fulvum* to successfully penetrate, colonize and reproduce on its host plant.

Race-specific incompatibility

Recognition of pathogens by resistant host plants leads to the induction of active plant defence and will result in incompatibility. The mechanism of pathogen recognition is still unknown. Several models have been proposed in which the recognition is mediated via resistance gene encoded receptors. In the last decade, avirulence genes and their direct or indirect products, responsible for the recognition by the resistant plant, have been isolated from different pathogens and characterized in detail (Keen, 1990).

The first bacterial avirulence gene was cloned in 1984 by mobilizing DNA clones from an avirulent race to a virulent race of *Pseudomonas syringae* pv. *glycinea* (Staskawicz *et al.*, 1984). This was the first example of a single gene from the pathogen that determines the outcome of a host - pathogen combination. Ever since many other bacterial avirulence genes have been isolated and characterized. Recognition by the host can be overcome by the pathogen due to (i) loss of the avirulence gene (Staskawicz *et al.*, 1984), (ii) inactivation by transposon insertion (Kearney *et al.*, 1988) and (iii) mutation within the avirulence gene (Kobayashi *et al.*, 1990).

The primary products of bacterial avirulence genes do not induce a hypersensitive response in resistant host plants, but are regulatory proteins or are involved in the formation of elicitors. There is one example where the avirulence gene *avrD* of *Pseudomonas syringae* pv. *tomato* is involved in the formation of a non-proteinaceous race-specific elicitor (Keen *et al.*, 1990). The direct interaction of the primary avirulence gene product with the host has been suggested for *avrBs3* of *Xanthomonas campestris* pv. *vesicatoria*. The predicted protein product of *avrBs3* consists of 17.5 nearly identical 34 amino acid repeat units (Bonas *et al.*, 1989). The number and type of repeats affect the avirulence spectrum, suggesting that the primary gene products function as elicitors by direct interaction with receptors in the resistant host plant (Herbers *et al.*, 1992).

The only examples of race-specific elicitors which are primary avirulence gene products are the capsid protein of tobacco mosaic virus (Knorr and Dawson, 1988), the necrosis-inducing peptides of *Rhynchosporium secalis* (Wevelsiep *et al.*, 1992) and the peptide

elicitors of *Cladosporium fulvum* (De Wit and Spikman, 1982; Scholtens-Toma and De Wit, 1988; Joosten *et al.*, in preparation).

Fungal avirulence genes have not been isolated by a shot-gun cloning approach as reported for bacterial avirulence genes. The large genome size of filamentous fungi and the low transformation frequency as compared to that of bacteria make this technique time consuming. The first fungal avirulence gene to be cloned, *avr9*, was isolated from *C. fulvum* by using amino acid sequence data of the race-specific peptide elicitor (Chapter 2 and 3). A second avirulence gene from *C. fulvum*, *avr4*, was cloned by a similar approach (Joosten *et al.*, in preparation). Recently, two avirulence genes of *Magnaporthe grisea* were isolated by map-based cloning (Valent *et al.*, 1993).

The studies on the avirulence gene *avr9* described in this thesis provide a picture of the events preceding the recognition of the AVR9 race-specific elicitor by the resistant host. Following penetration of the leaf through stomata the expression of the *avr9* gene is induced. *In vitro* experiments indicate that *avr9* is induced under conditions of nitrogen limitation, which might reflect growth conditions *in planta* (Chapter 6). The AVR9 precursor peptide of 63 aa is excreted to the intercellular space and the putative signal peptide of 23 aa is removed. The extracellular peptide of 40 aa is sequentially processed by fungal and plant proteases to a stable 28 aa peptide, which can accumulate to high concentrations in the apoplast of susceptible plants (Chapter 5). This mature peptide is an active elicitor of the hypersensitive response in resistant plants, although we do not exclude that the intermediate forms of 32, 33 and 34 aa are active elicitors as well. The primary function of the *avr9* gene for the pathogen itself is still unknown. Wild-type races of *C. fulvum* virulent on tomato genotype *Cf9* have lost the corresponding avirulence gene *avr9*. Preliminary studies indicate that disruption mutants lacking the *avr9* gene are still pathogenic on tomato indicating that *avr9* might be dispensible (chapter 4). The corresponding resistance gene *Cf9*, however, still proves to be stable in the tomato growing areas, suggesting that the races lacking *avr9* have a reduced fitness.

Race-specific incompatibility is depicted in the second model (Fig. 2) describing the interaction between a resistant tomato genotype and an avirulent race of *C. fulvum*. According to this model, expression of avirulence genes *in planta* is induced by factors and/or environmental stimuli from the plant. The gene products, the race-specific elicitors, are recognized by putative receptors present in the plant plasma membrane. Upon recognition, defence responses are triggered and as a result fungal growth is restricted at an early stage. The crucial factors in the complex defence response which are responsible for the ultimate restriction of fungal growth are not yet known. Most probably, a combination of different defence responses, including the hypersensitive response, will finally result in effective inhibition of fungal growth.

The fungal component involved in the recognition event inducing resistance, the race-specific elicitor, is now well documented and supports the gene-for-gene hypothesis. Recognition of race-specific elicitors induces active plant defence and thereby blocks the

basic ability of *C. fulvum* to successfully colonize tomato. The underlying mechanisms involved in recognition and subsequent signal transduction are, however, not yet understood. Recently, evidence was obtained for the involvement of G-protein mediated signal transduction following the recognition of a race-specific peptide elicitor of *C. fulvum* (Vera-Estrella *et al.*, 1993). The results suggest that a membrane bound receptor is coupled to a G-protein which is involved in the activation of phosphatases which in turn stimulate plasma membrane H^+-ATPases by dephosphorylation. Future research will tell us more about the structure of these receptors, the putative products of resistance genes. Whether the genes encoding receptors are indeed *Cf* resistance genes can only be proven by complementing *Cf*-less plants with these genes and score for resistance of these transformed plants to races with the complementary avirulence gene.

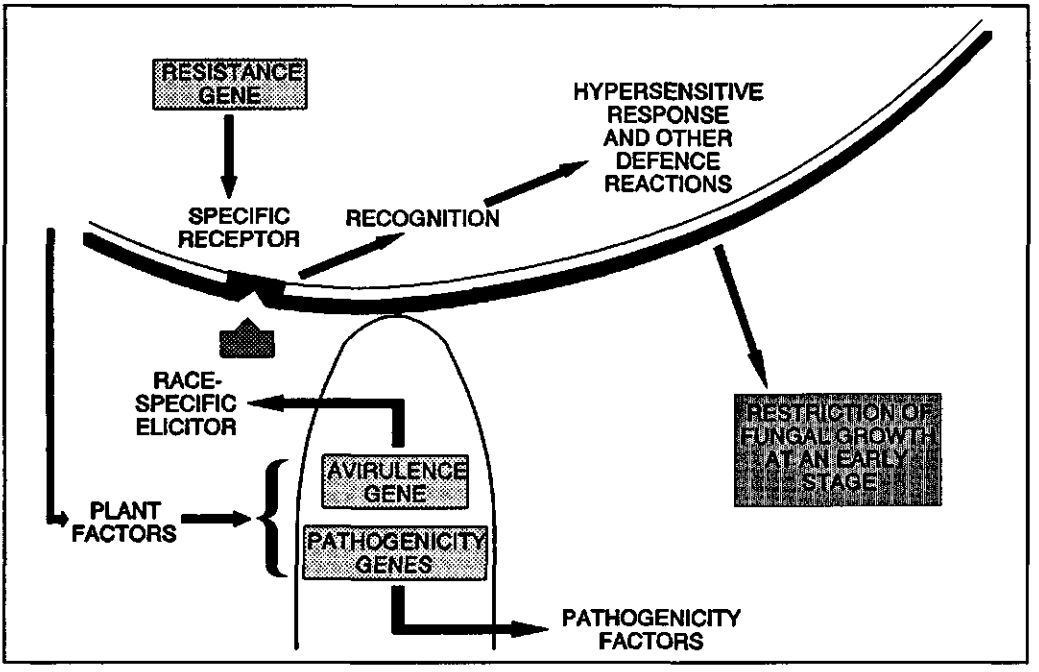

Figure 2. Schematic representation of events involved in race-specific incompatibility between avirulent races of *C. fulvum* and resistant tomato cultivars (details discussed in text; adapted from Van den Ackerveken and De Wit, 1993).

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Summary

The molecular understanding of host-pathogen interactions and particularly of specificity forms the basis for studying plant resistance. Understanding why a certain plant species or cultivar is susceptible and why other species or cultivars are resistant is of great importance in order to design new strategies for future crop protection by molecular plant breeding.

In this thesis molecular aspects of avirulence and pathogenicity of the tomato pathogen *Cladosporium fulvum* are described. The interaction *C. fulvum* - tomato is an excellent model system to study fungus - plant specificity as the communication between pathogen and plant is confined to the apoplast (intercellular space). The ability to obtain intercellular fluid from *C. fulvum*-infected tomato leaves enabled the isolation and characterization of plant and fungal compounds that might play an important role in pathogenesis and/or the induction of resistance. The purification and characterization of a race-specific peptide elicitor provides the basis for most of the experiments described in this thesis. This peptide was thought to be produced only by races of *C. fulvum* avirulent on tomato genotypes carrying the resistance gene *Cf9*, on which the elicitor induced necrosis.

Molecular aspects of avirulence of *C. fulvum* were first studied by the isolation and characterization of the cDNA encoding the AVR9 race-specific peptide elicitor (Chapter 2). The peptide was shown to be indeed produced by *C. fulvum*. Races virulent on tomato genotype *Cf9* lack the *avr9* gene and do not produce the peptide elicitor thereby evading recognition by tomato genotypes carrying the corresponding resistance gene *Cf9*. To prove that the *avr9* gene is a genuine avirulence gene, races virulent on tomato genotype *Cf9* were transformed with the cloned *avr9* gene (Chapter 3). The cultivar-specificity of the transformants was changed from virulent to avirulent on tomato genotype *Cf9*. The *avr9* gene can therefore be considered to be a genuine avirulence gene, the first fungal avirulence gene cloned. Additional proof for the role of the avirulence gene *avr9* in specificity was provided by the disruption of *avr9* in two races avirulent on tomato genotype *Cf9*, by gene replacement, resulting in transformants virulent on tomato genotype *Cf9* (Chapter 4).

The *avr9* gene encodes a 63 amino acids precursor protein. Removal of a signal peptide results in an extracellular peptide of 40 amino acids. Proteases of *C. fulvum* are involved in further processing this extracellular peptide by removal of N-terminal amino acids resulting in peptides of 32, 33 and 34 amino acids. Plant factors are responsible for further processing, resulting in the stable peptide elicitor of 28 amino acids (Chapter 5).

The avirulence gene *avr9* is highly expressed in *C. fulvum* while growing inside the tomato leaf. The expression of *avr9* is induced in *C. fulvum* grown *in vitro* under conditions of nitrogen limitation. The high expression of *avr9* in *C. fulvum* growing inside the tomato leaf might be caused by nitrogen limiting conditions in the apoplast (Chapter 6).

Pathogenicity of *C. fulvum* was studied at the molecular level by the isolation of two genes encoding extracellular proteins (ECPs). The *ecp*1 and *ecp*2 genes were isolated via the amino acid sequence of ECP1, and polyclonal antibodies raised against ECP2, respectively (Chapter 7). The expression of the *ecp* genes is highly induced *in planta* as compared to the *in vitro* situation. The availability of the cloned *ecp* genes now enables us to study the role and importance of these genes during pathogenesis by reporter gene analysis and gene disruption.

Two models describing the *C. fulvum*-tomato interaction are presented, dealing with basic compatibility and race-specific incompatibility, respectively (Chapter 8). The improved understanding of pathogen recognition can be exploited in future research to elucidate the role of putative receptors in the resistant plant involved in perception of elicitors and induction of active plant defence.

Samenvatting

De moleculaire kennis van plant-pathogeen interacties en in het bijzonder van specificiteit vormt de basis voor het onderzoek naar resistentie van planten. De wetenschap waarom een bepaalde plantesoort of cultivar vatbaar is en waarom een andere soort of cultivar resistent is, is van groot belang voor het ontwikkelen van nieuwe strategieën voor de gewasbescherming door middel van moleculaire plantenveredeling.

In dit proefschrift worden de moleculaire aspecten van avirulentie en pathogeniteit van het tomatepathogeen *Cladosporium fulvum* beschreven. De interactie *C. fulvum* - tomaat is een uitstekend modelsysteem voor het onderzoek naar schimmel - plant specificiteit, omdat de communicatie tussen pathogeen en plant beperkt is tot de apoplast (intercellulaire ruimte). De techniek om van *C. fulvum*-geïnfecteerde tomatebladeren de intercellulaire vloeistof te isoleren, maakte het mogelijk om schimmel- en plantfactoren te isoleren en karakteriseren die mogelijk een belangrijke rol spelen tijdens de pathogenese en/of de inductie van resistentie. De zuivering en karakterisering van een fysio-specifieke peptide elicitor vormen de basis voor de meeste van de in dit proefschrift beschreven experimenten. Dit eiwit werd verondersteld alleen geproduceerd te worden door fysio's van *C. fulvum* die avirulent zijn op tomaat-genotypen met het resistentiegen *Cf9*, waarop de elicitor necrose induceert.

De moleculaire aspecten van avirulentie van *C. fulvum* werden onderzocht door de opsporing en karakterisering van het cDNA coderend voor de fysio-specifieke elicitor AVR9 (Hoofdstuk 2). Het peptide bleek inderdaad door *C. fulvum* geproduceerd te worden. Fysio's die virulent zijn op tomaat-genotype *Cf9* missen het avirulentiegen *avr9* en produceren geen fysio-specifieke elicitor, waardoor ze de herkenning door tomaat-genotypen met het resistentiegen *Cf9* omzeilen. Om te bewijzen dat het *avr9* gen een echt avirulentiegen is, werden fysio's virulent op tomaat-genotype *Cf9* getransformeerd met het gekloneerde *avr9* gen (Hoofdstuk 3). De cultivar-specificiteit van de transformanten werd hierdoor veranderd van virulent naar avirulent op tomaat-genotype *Cf9*. Het *avr9* gen blijkt dus een echt avirulentiegen te zijn, het eerste gekloneerde avirulentiegen van een schimmel. Aanvullend bewijs voor de rol van het avirulentiegen *avr9* in schimmel - plant specificiteit werd verkregen door de disruptie van het *avr9* gen in twee fysio's avirulent op tomaat-genotype *Cf9*, door middel van vervanging van het *avr9* gen (gene replacement). Dit resulteerde in transformanten die virulent zijn op tomaat-genotype *Cf9* (Hoofdstuk 4).

Het *avr9* gen codeert voor een precursor eiwit van 63 aminozuren. Verwijdering van het signaalpeptide resulteert in een extracellulair peptide van 40 aminozuren. Proteases van *C. fulvum* zijn betrokken bij de processing van dit extracellulaire peptide door verwijdering van de N-terminale aminozuren resulterend in peptiden van respectievelijk 32, 33 en 34 aminozuren. Factoren van de plant zijn verantwoordelijk voor de verdere processing, resulterend in een stabiele peptide elicitor van 28 aminozuren (Hoofdstuk 5).

Het avirulentiegen *avr*9 komt sterk tot expressie in *C. fulvum* tijdens de kolonisatie van het tomateblad. De expressie van het *avr*9 gen wordt geïnduceerd in *in vitro* gekweekt *C. fulvum* mycelium onder stikstoflimiterende condities. De hoge expressie van *avr*9 in *C. fulvum* tijdens de kolonisatie van het tomateblad wordt mogelijk veroorzaakt door stikstoflimiterende condities in de apoplast (Hoofdstuk 6).

Pathogeniteit van *C. fulvum* is op moleculair niveau bestudeerd door de isolatie van twee genen die coderen voor extracellulaire eiwitten (extracellular proteins, ECPs). De *ecp*1 en *ecp*2 genen zijn achtereenvolgens geïsoleerd via de aminozuurvolgorde van ECP1, en polyklonale antilichamen tegen ECP2 (Hoofdstuk 7). De expressie van deze *ecp* genen in *C. fulvum* is sterk geïnduceerd *in planta* in vergelijking met de situatie *in vitro*. De beschikbaarheid van de gekloneerde *ecp* genen maakt het nu mogelijk om de rol en het belang van deze genen tijdens de pathogenese te bestuderen met behulp van reportergen analyse en gendisruptie.

Twee modellen voor de *C. fulvum* - tomaat interactie worden gepresenteerd, betrekking hebbend op respectievelijk basis compatibiliteit en fysio-specifieke incompatibiliteit (Hoofdstuk 8). De verkregen kennis met betrekking tot herkenning van pathogenen door de plant kan aangewend worden om inzicht te verkrijgen in de rol van mogelijke receptoren in de resistente plant die betrokken zijn bij de herkenning van elicitoren en de inductie van actieve afweer van de plant.

Account

Most of the results presented in this thesis have been published, or will be published in the near future. The contents of the preceding chapters have been based on most of these publications.

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Curriculum vitae

Augustinus Franciscus Johannes Maria van den Ackerveken werd op 21 april 1967 te Breda geboren. In 1984 behaalde hij het diploma HAVO aan de scholengemeenschap "Markenhage" te Breda. In hetzelfde jaar begon hij aan de opleiding tot botanisch laboratorium medewerker aan de toenmalige Rijks Hogere Agrarische School (de huidige Internationale Agrarische Hogeschool "Larenstein", afdeling laboratoriumonderwijs) te Wageningen. Na een stage op de toenmalige Stichting voor Plantenveredeling bij de afdeling Grassen en een afstudeeropdracht op de vakgroep Fytopathologie van de Landbouwuniversiteit te Wageningen werd het diploma voor botanisch laboratorium medewerker in 1988 behaald. Het afstudeerproject bij dr. P.J.G.M. de Wit werd vervolgens 6 maanden voortgezet middels een aanstelling als analist. Hierna heeft hij 6 maanden ervaring opgedaan in het moleculair genetisch onderzoek aan schimmels in de groep van dr. H.W.J. van den Broek op de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit te Wageningen. In augustus 1989 werd hij aangesteld als onderzoeker in opleiding (BION, NWO) bij de vakgroep Fytopathologie van de Landbouwuniversiteit. In de onderzoeksgroep van dr. P.J.G.M. de Wit was hij tot februari 1993 werkzaam aan het project getiteld: "Opsporing en klonering van genen die coderen voor fysio-specifieke elicitoren in de waardplant-schimmel interactie *Cladosporium fulvum*-tomaat". De resultaten van het gedurende deze periode uitgevoerde onderzoek zijn beschreven in dit proefschrift. Sinds februari 1993 is hij werkzaam als toegevoegd onderzoeker bij de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit.