The *Cladosporium fulvum* – tomato interaction:
physiological and molecular aspects of pathogenesis

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in het bijzonder de fysiologische aspecten.

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dr. H. C. van der Plas,
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van de Landbouwuniversiteit te Wageningen.

Het onderzoek beschreven in dit proefschrift werd verricht bij de vakgroep Fytopathologie van de Landbouwuniversiteit. Het onderzoek werd uitgevoerd in het kader van contract BAP-0074-NL van het Biotechnology Action Programme van de Europese Gemeenschap.

STELLINGEN

1. De door Camacho Henriquez en Sänger gevolgde methode voor het zuiveren van het "pathogenesis-related" eiwit P14 is niet doordacht en heeft geen zuiver eiwit opgeleverd.

   Camacho Henriquez en Sänger, Arch Virol (1984) **81**: 263-284.
   Dit proefschrift.

2. Doordat de "pathogenesis-related" (PR) eiwitten van tabak veelal worden geanalyseerd op basische gels en die van tomaat op zure, lijken de verschillen tussen de in tabak en tomaat geïnduceerde PR eiwitten groter dan ze in werkelijkheid zijn.

   Van Loon, Plant Mol Biol (1985) **4**: 111-116.
   De Wit *et al.*, Physiol Mol Plant Pathol (1986) **29**: 159-172.
   Dit proefschrift.

3. Om de problemen zoals die zich nu voordoen met de nomenclatuur van de "pathogenesis-related" (PR) eiwitten van tabak te voorkomen, wordt het de hoogste tijd de naamgeving van de PR eiwitten van tomaat te standaardiseren.

4. Door er op te wijzen dat pathogenen en ethyleen in boon (*Phaseolus vulgaris*) identieke chitinases en 1,3-ß-glucanases induceren, suggereren Mauch en Staehelin ten onrechte dat met ethyleen behandeld weefsel een algemeen bruikbaar modelsysteem vormt voor *in situ* lokalisatie van deze enzymen.

   Mauch en Staehelin, The Plant Cell (1989) **1**: 447-457.

5. De door Lucas en medewerkers gepubliceerde aminozuurvolgorde van het "pathogenesis-related" eiwit P14 is niet volledig.

   Lucas *et al.*, The EMBO J (1985) **11**: 2745-2749.
   Dit proefschrift.

6. Het mutagene effect van milde sonicatie als alternatieve methode voor transformatie van plantprotoplasten, wordt door Joersbo en Brunstedt onderschat.

   Joersbo en Brunstedt, Plant Cell Reports (1990) **9**: 207-210.

7. Degelijk "eiwitwerk" vormt vaak de basis voor succesvol moleculair biologisch onderzoek.

8. Hoewel men bij de aanbeveling gebruik te maken van het 'yeast artificial chromosome' (YAC) kloneringssysteem vaak benadrukt dat DNA fragmenten van 500 Kb en groter kunnen worden gekloneerd, bevatten de meeste van de tot nu toe geconstrueerde YAC-banken slechts DNA inserties van 75 tot 200 Kb.

9. Het is duidelijk dat door de prijsverhogingen van én het autorijden én het openbaar vervoer, de overheid eigenlijk maar één zaak wil stimuleren, namelijk: de groei van de schatkist.

10. Het valt te betwijfelen of het plaatsen van een kunstwerk bestaande uit grote doofpotten bij het bestuurscentrum van de Landbouwuniversiteit stimulerend werkt op de communicatie met de vakgroepen.

Stellingen behorend bij het proefschrift "The *Cladosporium fulvum*-tomato interaction: physiological and molecular aspects of pathogenesis."
Wageningen, 31 mei 1991 Matthieu H.A.J. Joosten

voor mijn ouders
en voor Titia

VOORWOORD

Toen ik mij bij de vakgroep Fytopathologie ging oriënteren op de mogelijkheden die bestonden voor het uitvoeren van een afstudeeropdracht, kwam ik al snel in de ban van het onderzoek aan plant-pathogeen interacties dat op deze vakgroep wordt verricht. Ik besloot bij Pierre de Wit aan de interactie tussen de schimmel *Cladosporium fulvum* en tomaat te gaan werken. De mogelijkheid om deze interactie het onderwerp te maken van een promotieonderzoek greep ik met twee handen aan. Gedurende de drie en een half jaar waarin ik als promovendus werkzaam was heb ik de opbloei van de onderzoeksgroep van Pierre de Wit meegemaakt. In 1986 waren we met twee promovendi, twee analisten en één electroforese apparaat (weet je nog Grardy ?). Nu is het uitgegroeid tot een groep bestaande uit een vaste medewerker, twee post-docs (waarvan ik er nu een ben), twee promovendi en twee analisten. Pierre heeft inmiddels het hoogleraarschap van professor Dekker overgenomen. Door financiële ondersteuning van het bedrijfsleven en de EG is er aan apparatuur en chemicaliën geen gebrek meer.

Ik heb gedurende mijn promotieonderzoek steeds met zeer veel plezier gewerkt en geen moment getwijfeld aan de onbegrensde mogelijkheden van het onderzoek aan de *C. fulvum* - tomaat interactie. Voor het voorspoedige verloop van het onderzoek ben ik ten eerste Pierre de Wit erg dankbaar. Zijn niet aflatende enthousiasme en energie hebben mij ook in moeilijkere tijden op de been gehouden. Door zijn vele contacten met andere onderzoeksgroepen, zijn fabuleus geheugen en het snel inspelen op nieuwe ontwikkelingen, is hij steeds in staat geweest het onderzoek up to date te houden.

Verder ben ik alle medewerkers van de vakgroep die direkt of indirekt hebben bijgedragen tot de totstandkoming van dit proefschrift zeer dankbaar. Ook bedankt voor de goede, ontspannen werksfeer op het lab. Van mijn naaste collega's wil ik Jan van Kan en Guido van den Ackerveken met name noemen. Jan, dank je voor je verwoede pogingen mij wat van de moleculaire biologie bij te brengen. Ik hoop dat het niet helemaal tevergeefs is geweest. Verder kan ik het erg waarderen dat je mij hebt toevertrouwd aan je studente, Titia Sijen, uit te leggen hoe je eiwitten electroforeert. Ik heb nooit geweten dat het zo'n gevolgen kon hebben. Guido, dank je voor het, al dan niet vrijwillig, afstaan van steriele pipetpunten, epjes en stockoplossingen. Ook nog bedankt voor je tegenstand op de squashbaan (al vind ik wel dat je vaak geluk hebt en onhandig met rackets omgaat).

Verder ben ik natuurlijk de studenten die hun deel hebben bijgedragen aan het onderzoek dankbaar. Albert Kerssies, Ineke Booij, Joyce de Wit, Jaap bij de Vaate, Birgit Loos, Philip Pasmans en Cécile Bergmans, jullie wisten mij vaak te stimuleren en risicovolle ideën toch tot een goed einde te brengen.

Buiten het lab werd ik moreel bijgestaan door de leden van de 'eetgroep'. Ad, Guido, Hans, Mirjam, Nelleke, Olga en Theo bedankt voor de voedzame maaltijden en de vruchtbare discussies, ze hebben mij gesterkt bij het schrijven van dit proefschrift. Ook de 'werkbesprekingen' met Raymond van Daelen bij H41 hebben mij steeds weer nieuwe inspiratie gegeven. Raymond, al spraken we niet zo vaak over het werk en was het de volgende ochtend wel eens moeilijk, die donderdagavonden had ik toch niet graag willen missen. Dank aan het personeel van H41, de De Koninckjes waren altijd prima op temperatuur!

Speciale dank ben ik verschuldigd aan het LEB-Fonds, dat door een financiële bijdrage de kosten van het drukken van het proefschrift een stuk dragelijker heeft gemaakt.

En dan natuurlijk, lest best, Titia. Toen jij bij Jan kwam werken had het lab binnen een week in de gaten dat er tussen ons sprake was van basiscompatibiliteit. Hoewel de incubatietijd wat lang was, hebben zich uiteindelijk mooie symptomen ontwikkeld. Ondanks dat je pas aan het eind van mijn promotieonderzoek op het toneel verscheen en al snel weer richting Berlijn vertrok, ben je toch een grote steun voor mij geweest en weet ik zeker dat je dat nog heel lang zult blijven!

Matthijn

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. PLANT-PATHOGEN INTERACTIONS

1.1 GENERAL

Generally four relationships can be distinguished between plants and their surrounding micro-organisms:

(i) Zero-relationship. There is no obvious interaction between plant and micro-organism.

(ii) Non-host relationship (basic incompatibility). Plants are not infected by the majority of micro-organisms due to the presence of a number of preformed (passive) defense factors such as physical barriers or antimicrobial compounds that prevent successful colonization.

(iii) Beneficial relationship. Examples are interactions between legumenous plants and nitrogen-fixing bacteria or interactions between several plant species and mycorrhizal fungi.

(iv) Pathogenic relationship (basic compatibility). The micro-organism is a pathogen that either is able to kill host cells before a defense reaction can occur or is specialized in avoiding the elicitation of resistance mechanisms of the plant, resulting in successful colonization of the host. In the latter case the pathogen grows biotrophically and avoids serious host damage, at least in the early stages of infection.

In biotrophic and obligate relationships, physiological specialization has frequently been observed. Different host cultivars show specific responses to distinct races of a pathogen. Molecular signals between plant and pathogen determine whether the interaction will be incompatible or compatible.

In an incompatible interaction (host resistant, pathogen avirulent) early molecular recognition of the pathogen attempting to penetrate the host occurs, followed by a rapid activation of plant defense reactions.

In a compatible combination (host susceptible, pathogen virulent) no molecular recognition and elicitation of plant defense responses occur, allowing the pathogen to successfully colonize its host.

1.2 THE GENE-FOR-GENE HYPOTHESIS

The phenomenon of race-specific resistance is frequently observed among biotrophic and obligate parasites. From genetic studies on several host-pathogen interactions it appeared that resistance against particular races of a pathogen usually inherits as a dominant factor. Similarly, avirulence within the pathogen usually inherits in a dominant way. Race-specific resistance is believed to involve a specific interaction between products of avirulence genes and products of corresponding resistance genes, resulting in initiation of a hypersensitive response and a number of other defense responses (15). This so-called gene-for-gene concept is thought to hold for many interactions in which biotrophic or obligate parasites are involved (5) and implies that for each gene conditioning avirulence in the pathogen there is a corresponding gene conditioning resistance in the host.

The exchange of information between host and pathogen, inducing complex responses that determine the outcome of an interaction, is summarized in Figure 1. The different items shown in the figure will be discussed in the following section. The discussion will be limited to interactions between plants and pathogenic fungi.

2. INTERACTIONS BETWEEN PLANTS AND PATHOGENIC FUNGI

2.1 FUNGAL PATHOGENICITY FACTORS

Fungal pathogens carry various pathogenicity genes that encode a wide range of pathogenicity factors required to obtain basic compatibility and to successfully colonize the host. Besides the ability to produce cutinases or cell wall degrading enzymes or to detoxify phytoalexins (of which the results of some recent studies will be discussed), the production of toxins, proteases, suppressors of plant defense reactions or special penetration structures can be considered as important pathogenicity factors (for recent reviews see 20, 28).

Plant pathogenic fungi that are able to penetrate plant organs directly have to pass the cuticle, a layer that mainly consists of cutin, an insoluble, hydrophobic biopolyester that is composed of hydroxy- and hydroxyepoxy fatty acids. Dickman and co-workers (14) have shown that the production of cutinases,

enzymes that are potentially able to degrade the cutin barrier, can be considered as an important pathogenicity factor for those fungi. The cutinase gene from *Fusarium solani* f. sp. *pisi* has been cloned and a plasmid containing the structural gene and extensive portions of the 5'- and 3'-flanking regions has been

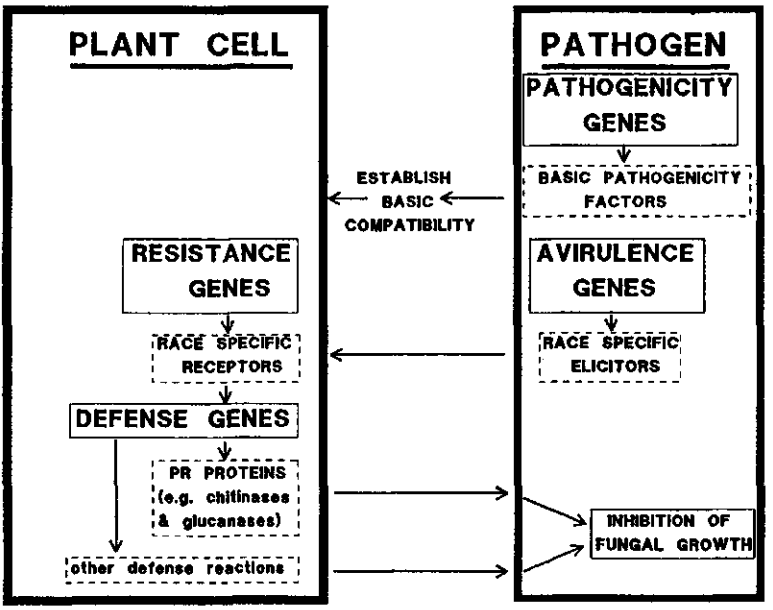

Figure 1. Schematic representation of the interplay between host and pathogen. Signals and responses determine the outcome of the interaction. The several items in the figure are discussed in section 2.

constructed. When the gene was introduced into *Mycosphaerella* spp., a fungus that is only able to infect wounded papaya fruits, transformants were obtained that expressed the cutinase gene and consequently were able to infect intact papaya fruits. Antibodies raised against the cutinase from *F. solani* f. sp. *pisi* prevented lesion formation by the transformants. When the cutinase gene was introduced into a mutant of *F. solani* f. sp. *pisi* that did not produce cutinase and was not able to penetrate intact pea stems, its pathogenicity was restored (19).

Fungi that are able to degrade the plant cell wall underlying the cutin layer produce an array of highly specific cell wall-degrading enzymes (CWDEs). These enzymes can also be considered as pathogenicity factors. Of the CWDEs, the pectin degrading enzymes, such as pectate lyase (PL) and polygalacturonase (PG), have been studied most intensively. Although there is circumstantial evidence indicating that these pectolytic enzymes are determinants of pathogenicity (for reviews see 20, 28), their actual role in pathogenesis remains to be determined. Molecular genetic techniques that permit a direct evaluation of the role of pectolytic enzymes during the course of the disease are now being developed for non-pathogenic and pathogenic fungi. Dean and Timberlake (7, 8) purified PL produced by *Aspergillus nidulans* after being cultured on minimal medium containing 1 % polygalacturonic acid. PL-cDNA clones were isolated by screening an expression library with antibodies raised against the purified protein and it was shown that the gene encoding PL is present as a single copy in the genome. The PL gene (*pel*A) was isolated from an *A. nidulans* cosmid bank and targeted mutation of the gene blocked PL production but did not affect the production of PG and growth on polygalacturonic acid. PL expression was regulated at the level of mRNA accumulation; transfer of the fungus from medium containing glucose as carbon source to medium containing polygalacturonic acid or polygalacturonic acid plus glucose resulted in the accumulation of a transcript of 1300 nucleotides in length that hybridized with the *pel*A cDNA clone. When glucose or acetate was present as sole carbon source, no *pel*A mRNA was detected in the mycelium. The release of glucose and other sugars from wounded tissues could probably inhibit the production of pectolytic enzymes, resulting in limited disease development. Future studies on fungal pathogens will reveal whether similar regulatory control mechanisms occur for those fungi while growing in host tissues.

The importance of phytoalexin detoxification for pathogenicity has recently been reviewed by Van Etten *et al* (32). Most studies in this area have been focused on pisatin tolerance and detoxification in pea diseases. Studies of Cruickshank (4) revealed that tolerance to pisatin might be an important factor for pathogenicity on pea; only five of 50 fungal isolates (representing 45 species) that were tested were tolerant to pisatin and were pathogens of pea. Subsequent studies by other researchers showed that various pea pathogens were able to metabolize pisatin (for references see 32) and it was shown that the first step in

this degradation consists of a demethylation of the phytoalexin to a less toxic compound (31). To directly test the importance of pisatin tolerance and detoxification for pathogenicity of a fungal pea pathogen, the effect of the presence and absence of phytoalexin detoxification within a single fungal species was studied. From the ascomycete *Nectria haematococca* isolates were obtained that were not able to demethylate pisatin (Pda^-) and were non-pathogenic on pea. Crosses between Pda^+ and Pda^- isolates revealed that all progeny able to infect pea were Pda^+ and tolerant to pisatin (for references see 32). A phytoalexin-detoxifying gene from *N. haematococca* was isolated by transforming *Aspergillus nidulans* with fragments of DNA isolated from *N. haematococca* (35). Transformants expressing the gene were able to demethylate pisatin and showed an increased tolerance to the phytoalexin. Transformation of Pda^- isolates of *N. haematococca* with the gene restored their virulence towards pea, while expression of the gene in the maize pathogen *Cochliobolus heterostrophus* increased the size of the lesions that were formed by this fungus on pea (for references see 32).

2.2 FUNGAL AVIRULENCE GENES/HOST RESISTANCE GENES

A major implication of the gene-for-gene hypothesis is that plant resistance is the result of the interaction of molecular signals encoded by fungal avirulence genes with products encoded by host resistance genes. It is envisaged that fungal avirulence genes encode race-specific elicitors that bind to cultivar-specific receptors which are the putative products of corresponding resistance genes of the host (specific elicitor-specific receptor model) (13). Following elicitor binding, intracellular signalling events occur resulting in activation of host defense genes.

The interaction between the obligate parasite *Bremia lactucae* and lettuce is a model of which the genetic and to some extent the molecular aspects are intensively studied. The major determinants of specificity in this plant-pathogen system are involved in a gene-for-gene interaction (17); 13 dominant avirulence genes in the fungus match 13 dominant resistance genes in the host. One of the goals of the research group of Michelmore (17) is to clone and characterize avirulence genes from *B. lactucae*. Avirulence genes will be isolated by chromosome walking from restriction fragment length polymorphism (RFLP) markers linked to these genes. The development of a transformation system for

the fungus is necessary to eventually prove that the cloned genes are indeed avirulence genes.

The gene-for-gene interaction between the biotrophic fungal parasite *Cladosporium fulvum* and tomato is also a model system that is intensively studied (see section 3). The putative product of avirulence gene *avr9* of *C. fulvum* has been purified and its amino acid sequence has been determined (27). A cDNA and genomic clone containing *avr9* have been obtained (33) and currently virulent races of *C. fulvum* are being transformed with these clones (for further discussion see chapter 4). Further research might eventually lead to the isolation and characterization of the corresponding resistance gene of the host.

2.3 PLANT DEFENSE GENES

Active resistance against certain races of a fungus is often associated with a hypersensitive response that involves rapid death of the cells that are in direct contact with the penetrating fungus and the activation of a number of host defense genes. Inducible defense responses that will be briefly discussed here include the deposition of hydroxyproline rich glycoproteins (HRGPs, also referred to as extensin) and lignin in host cell walls and the production of hydrolytic enzymes, such as 1,3-ß-glucanases and chitinases (for detailed reviews see 6, 13, 15).

The accumulation of HRGPs has been suggested to be part of the active defense mechanism of several plant species (for a recent review see 29). Mazau and Esquerré-Tugayé (23) showed that accumulation of the glycoprotein in several dicotelydons was triggered by viral, bacterial and fungal infections. Upon inoculation with *Cladosporium cucumerinum*, cultivars of cucumber resistant to the fungus showed a faster increase in cell wall hydroxyproline content than susceptible cultivars (16). Hybridization of blots containing mRNA from healthy or infected melon plants with a genomic HRGP clone of carrot revealed five mRNA species in infected plants (25). Induction of the mRNAs occurred 3 to 4 days after inoculation, consistent with the time course of accumulation of HRGPs in the cell walls. Immunocytochemical studies on the localization of HRGPs in melon and bean infected by bacteria or fungi, using antibodies raised against a HRGP of melon, indicated that HRGPs accumulate at sites where growth of the microbe is inhibited (24). The localization of these glycoproteins supports their

possible involvement in agglutination and immobilization of bacteria and strengthening of the plant cell wall.

Lignification might also be an important factor in active plant defense (36). In the cultivar-specific resistance expressed by wheat to the leaf rust fungus *Puccinia recondita* f.sp. *tritici*, lignification is thought to play an important role (30). In wheat cv Thew, carrying the Lr20 allele for resistance, substantial lignification occurred in collapsed mesophyll cells surrounding fungal colonies that developed after inoculation with an avirulent race of *P. recondita* f. sp. *tritici*. At the same time fungal growth was inhibited. In wheat carrying the Lr28 allele, resistance was expressed by the inhibition of the development of haustoria in the mesophyll cells that were penetrated. Possibly phenolic precursors of lignin accumulated in the penetrated cells or haustoria became lignified.

Besides the accumulation of proteins that were shown to have a specific biological function, many proteins of unknown function are synthesized as a result of the activation of host defense genes. These proteins, which can accumulate in very large amounts, are referred to as "pathogenesis-related" (PR) proteins (for recent reviews on PR proteins see 1, 2, 34). Only recently (see also this thesis) a biological function for some of these PR proteins became apparent. Legrand and co-workers (21) purified two acidic and two basic chitinases from a homogenate of TMV-infected tobacco leaves and identified the two acidic members as the earlier described PR proteins PR-P and -Q. The two basic chitinases did not correspond to earlier described PR proteins of tobacco. Similarly, the acidic tobacco PR proteins PR-2, -N and -O were identified as 1,3-ß-glucanases (18). Both chitinases and 1,3-ß-glucanases are hydrolytic enzymes that are potentially able to hydrolyze fungal cell walls and therefore might inhibit fungal growth (22, 26).

3. THE TOMATO-*CLADOSPORIUM FULVUM* INTERACTION

Cladosporium fulvum (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) is a fungal pathogen that causes leaf mold of tomato (*Lycopersicon esculentum* Mill.). Under conditions of high humidity the disease can cause severe economic losses. The fungus probably originates from South America (3). In Europe the disease was first described in England in 1883. *C. fulvum* is a fungus that penetrates the

leaves through stomata and colonizes the intercellular spaces between the leaf mesophyll cells of susceptible tomato cultivars without causing visible damage initially. The biotrophically growing pathogen remains confined to the apoplast during the main part of its life cycle and no haustoria are formed. Once the intercellular spaces are colonized, the fungal mycelium emerges from stomata and conidiophores are produced. Eventually the colonized tomato leaves are killed by a general stress and non-functioning of stomata. Under natural conditions the infection cycle takes two to three weeks.

Though several resistance genes (obtained from various *Lycopersicon* species) have been introduced into a number of tomato cultivars, new races of *C. fulvum* were able to overcome resistance sooner or later. At present many genes for resistance have been introduced into near-isogenic lines of the tomato cultivar Moneymaker. These lines give a clear differential response to the presently known races of *C. fulvum*. Table I shows that the interaction between tomato and *C. fulvum* is a typical gene-for-gene relationship. The outcome of the interaction (incompatible, the tomato cultivar is resistant or compatible, the tomato cultivar is susceptible) can be predicted according to the combination of the resistance genes present in the tomato cultivar and the avirulence genes present in the race of *C. fulvum*.

Table I. Differential interactions between various races of *Cladosporium fulvum* and near-isogenic lines of tomato cultivar Moneymaker with different genes for resistance to *C. fulvum*.

		Races of *Cladosporium fulvum*				
		0	4	5	2 4 5	2 4 5 9
Tomato cultivars	Resistance genes	Genotypes of races				
		$A_2A_4A_5A_9$	$A_2a_4A_5A_9$	$A_2A_4a_5A_9$	$a_2a_4a_5A_9$	$a_2a_4a_5a_9$
Moneymaker Near-isogenic line	none	C	C	C	C	C
Cf2 Near-isogenic line	Cf2	I	I	I	C	C
Cf4 Near-isogenic line	Cf4	I	C	I	C	C
Cf5 Near-isogenic line	Cf5	I	I	C	C	C
Cf9	Cf9	I	I	I	I	C

In summary there are several reasons why the interaction between tomato and *C. fulvum* is an ideal model system to study recognition events between plant and pathogen:

(i) *C. fulvum* is a biotroph that colonizes the apoplastic area between tomato leaf mesophyll cells without forming haustoria and without damaging the cells initially. Thus, the exchange of molecular signals between host and pathogen remains confined to the apoplast, a region that can be readily explored by the isolation and analysis of apoplastic washing fluids.

(ii) The various races of *C. fulvum*, carrying the different avirulence genes, can easily be grown *in vitro*.

(iii) Several near-isogenic lines of the tomato cultivar Moneymaker that carry the different resistance genes are available.

(iv) Several resistance genes (Cf2, Cf4, Cf5, Cf9) give absolute protection against races of *C. fulvum* carrying the corresponding avirulence genes, suggesting that resistance is based on only one or a few factors that arrest fungal development.

4. LONG TERM STRATEGIES OF THE RESEARCH ON THE TOMATO-*C. FULVUM* INTERACTION

The ultimate aim of the research on the tomato-*C. fulvum* model system is the elucidation of the molecular basis of the gene-for-gene interaction. As the outcome of the interaction between host and fungus is probably defined by the interaction between products encoded by fungal avirulence genes and host resistance genes, characterization of these products and their encoding genes is one of the main objectives of the research.

In the incompatible interaction, after molecular recognition of the penetrating fungus by the plant, host defense reactions are activated and growth of the fungus is blocked. Analysis of the various responses of the host may reveal the basis of resistance and give more insight in how colonization of the host tissue is prevented.

In the compatible interaction, abundant colonization of the apoplast of the tomato leaf takes place without causing damage to the mesophyll cells initially.

Identification of basic pathogenicity factors which enable *C. fulvum* to successfully colonize tomato is object of study as well.

5. AIM AND OUTLINE OF THE PRESENT STUDY

The present study was undertaken to continue the various lines of physiological studies that were initiated by De Wit and co-workers (9, 11, 12) and to create a basis for a molecular approach to the different aspects of pathogenesis.

At the time of commencement of this research project, it had been shown that a protein, probably of fungal origin, accumulated in the apoplast of *C. fulvum*-infected tomato leaves and appeared to be specific for compatible interactions (12). There was evidence for the presence of race-specific elicitors in apoplastic fluids isolated from compatible *C. fulvum*-tomato interactions (9) and one race-specific elicitor, the putative product of avirulence gene *avr9*, had been purified and characterized (10, 27). Time course experiments had revealed that in the apoplast of *C. fulvum*-infected tomato leaves several PR proteins were present that accumulated more rapidly in the incompatible interaction than in the compatible one, suggesting that they are somehow associated with active host defense (11, 12).

In chapter 2 the purification and preliminary characterization of a protein that could be regarded as a basic pathogenicity factor is described. The protein was shown to be specific for compatible *C. fulvum*-tomato interactions and its origin and function are discussed.

Chapter 3 deals with changes in the carbohydrate pool present in tomato leaves with regard to a compatible and an incompatible interaction between tomato and *C. fulvum*. In a compatible interaction the invading fungus is able to hydrolyze apoplastic sucrose and to convert the resulting hexoses, glucose and fructose, into mannitol. In incompatible interactions no functional nutritional relationship between plant and fungus is established as a result of plant defense responses.

Chapter 4 describes the partial purification of a race-specific elicitor; the putative product of the avirulence gene *avr4* of *C. fulvum*. The fraction containing the race-specific elicitor specifically induced necrosis, chlorosis, epinasty and accumulation of PR proteins, supporting the hypothesis that the

elicitor is the molecular signal of the fungus that induces HR and other defense responses in tomato cultivars carrying resistance gene Cf4.

Further research on the correlation between the accumulation of apoplastic PR proteins and active host defense resulted in the identification of several PR proteins as 1,3-ß-glucanases and chitinases (chapter 5). The subcellular localization of these hydrolytic enzymes in tomato leaves infected by *C. fulvum* is described in chapter 6. It was shown that in the incompatible interaction chitinases and 1,3-ß-glucanases accumulate at the site of penetration by the fungus and bind to the hyphal wall.

In chapter 7 the purification and serological characterization of three basic 15 kD tomato PR proteins of unknown function are described. Two serologically related isomers of P14, one of the most abundantly occurring PR proteins of tomato, were identified (P4 and P6) and were shown to be related to the tobacco PR-1 protein family. The third PR protein (P2) was found to be serologically related to PR-R of tobacco. Chapter 8 describes the cloning and characterization of a cDNA encoding P6, the major component of P14. The cDNA clone contains an open reading frame of 477 nucleotides, encoding a signal peptide of 24 amino acids and the mature P6 protein of 135 amino acids in length.

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CHAPTER 2

ISOLATION, PURIFICATION AND PRELIMINARY CHARACTERIZATION OF A PROTEIN SPECIFIC FOR COMPATIBLE *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)-TOMATO INTERACTIONS

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Isolation, purification and preliminary characterization of a protein specific for compatible *Cladosporium fulvum* (syn. *Fulvia fulva*)–tomato interactions

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The interaction between *Cladosporium fulvum* and tomato has been used as a model system to study the accumulation of host-, pathogen- and interaction-specific proteins in leaf apoplastic fluids from compatible and incompatible combinations. Electrophoresis of apoplastic fluids under low pH, non-denaturing conditions revealed one protein which was present in all compatible interactions studied, but not in incompatible interactions nor in uninoculated controls.

Purification of this protein from the apoplastic fluids from several compatible interactions was achieved by ion exchange chromatography on CM-Sephadex followed by chromatofocusing. The purified protein migrated on SDS polyacrylamide gels as one band with an estimated molecular mass of 14 kD. Antibodies obtained by injecting the purified protein, bound to nitrocellulose, into rabbits had high affinity for the protein on Western blots and little or no interactions with other protein bands. In compatible *C. fulvum*–tomato interactions the protein could be detected in apoplastic fluid 8 days after inoculation. The protein was not detected in the mycelium or culture filtrates obtained from *C. fulvum* grown in culture, nor in apoplastic fluids from tomato leaves inoculated with the tomato strain of *Phytophthora infestans*. Furthermore, it was not detectable in old tomato leaves.

The possible origin of the protein and its function in basic compatibility, are discussed.

INTRODUCTION

The interaction between *Cladosporium fulvum* (syn. *Fulvia fulva*) and tomato has frequently been used as a model system for the study of recognition events in a gene-for-gene interaction between host plant and pathogen [*6–12, 18*]. Near-isogenic lines of tomato carrying resistance genes Cf2, Cf4, Cf5 or Cf 9 show clear differential responses to races 0; 2; 4; 5; 2.4; 2.4.5 and 2.4.5.9 of *C. fulvum* developing either a compatible or an incompatible reaction in response to infection by these races [*1,5*].

The fungus colonizes the intercellular space between mesophyll cells of tomato leaves and is confined to the apoplast [*5, 19, 20*]. The apoplast of the infected leaf may contain substances constitutively produced by the healthy plant, substances specifically induced in the plant by the fungus, substances of fungal origin of which some might be important for the establishment of basic compatibility, and substances which are specifically produced by certain races of the fungus which might play a role in race- or cultivar-specific resistance. These latter substances are also known as race-specific elicitors.

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TABLE 1
Differential interactions between races 4, 5 and 2.4.5.9 of Cladosporium fulvum *and the tomato cultivars used in this study*

Tomato cultivars	Genes for resistance	Races of *Cladosporium fulvum*		
		4	5	2.4.5.9
Near isogenic line Cf4 of Moneymaker	Cf4	C*	I	C
Near-isogenic line Cf5 of Moneymaker	Cf5	I	C	C

*C = compatible interaction; I = incompatible interaction

Fungus-mediated accumulation of pathogenesis-related (PR) proteins has been reported to occur in the apoplastic fluid of *C. fulvum* infected tomato leaves [*3, 6, 10*]. Also, race-specific elicitors of necrosis have been isolated from apoplastic fluids obtained from compatible interactions between *C. fulvum* and tomato [*6, 9, 11, 12, 17*].

Further analysis of apoplastic fluids by polyacrylamide gel electrophoresis under low pH, non-denaturing conditions revealed a soluble protein, previously designated P1 [*6*] which was present in all compatible interactions but not detectable in apoplastic fluids from incompatible interactions, nor in uninoculated controls. The common occurrence of this protein in compatible interactions suggests that it might be an important factor of basic compatibility [*16, 18*]. Here we report the purification and characterization of this compatible interaction-specific protein. The origin and possible role of this protein are discussed.

MATERIALS AND METHODS

Plants, fungi and inoculation

The various tomato cultivars containing different genes for resistance to *Cladosporium fulvum* Cooke (syn. *Fulvia fulva* (Cooke) Cif) were grown as described by de Wit & Flach [*4*].

Cladosporium fulvum was grown on potato dextrose agar (PDA) or in liquid B5-medium in shake culture [*8*]. The differential interactions between the tomato cultivars and the races of *C. fulvum* used in this study are listed in Table 1.

Phytophthora infestans (Mont.) de Bary (tomato strain) was subcultured as described by de Wit *et al.* [*6*]. The fungus was stored, as sporangia, in liquid nitrogen.

Tomato cultivars were inoculated with conidial suspensions of the various races of *C. fulvum* as described by de Wit [*5*]. Detached compound tomato leaves, inserted into florists' foam oases saturated with water, were inoculated with *P. infestans* by placing 10-µl drops of sporangial suspensions (about 10^4 ml^{-1}) on the lower surface of the leaflets. After inoculation the leaves were incubated in boxes maintained at 100% RH and a temperature of 18 °C.

Isolation of apoplastic fluids

For the time-course experiments, apoplastic fluids from compatible and incompatible interactions of *C. fulvum* and tomato were obtained at various times after inoculation.

Leaves were harvested at random and the intercellular spaces were washed by infiltrating the entire leaflet with distilled water *in vacuo*, followed by centrifugation, according to the method of de Wit & Spikman [*9*]. Apoplastic fluids from tomato leaves infected with *P. infestans* were obtained 6 days after inoculation.

Preparation of tomato leaves and mycelial extracts

After removal of the main vein, tomato leaves inoculated with *C. fulvum* were homogenized in a mortar with water (4·0 ml g^{-1} fresh weight) and sand. The homogenate was centrifuged for 15 min at 8000 ***g***, followed by 30 min at 35 000 ***g*** and the supernatant was collected and analysed by PAGE.

Fungal mycelia of races 4 and 5, grown for 7 days in liquid shake cultures were harvested and homogenized separately in a mortar with sand and water (6·0 ml g^{-1} fresh weight). The homogenates were centrifuged for 10 min at 1500 ***g***, followed by 15 min at 35 000 ***g*** and the resultant supernatants were collected and analysed by PAGE.

Protein determinations

Protein determinations were carried out according to Bradford [*2*], with bovine serum albumin (BSA) as a standard.

Purification of the compatible interaction specific protein (P1)

About 400 ml of apoplastic fluid originating from a compatible *C. fulvum*–tomato interaction was freeze-dried and 15 ml of 25 mM sodicum acetate buffer, pH 4·5, was added to the resulting residue. After thorough mixing and centrifugation for 10 min at 1500 ***g***, a clear supernatant was obtained which was applied to a CM-Sephadex C-25 column (1·3 × 30·0 cm, 10 ml bed volume), pre-equilibrated with 25 mM sodium acetate buffer, pH 4·5. The column was washed with 150 ml of 25 mM sodium acetate buffer, pH 4·5, containing 0·15 M NaCl at a flow rate of 10 ml h^{-1}, and then eluted with a linear gradient of 0·15 to 0·5 M NaCl in buffer the same for 4 h at a flow rate of 10 ml h^{-1}. The eluate was monitored for absorbance at 280 nm for protein and fractions of 1·0 ml were collected. The fractions containing proteins were dialysed against water (Spectro/Por tubing; cut off 3500 D), freeze-dried and further analysed by PAGE.

Fractions containing P1 were combined and subjected to chromatofocusing. Combined fractions were freeze-dried and the residue, after dissolving in 20 ml 25 mM imidazole–HCl buffer, pH 7·4, was applied to a Polybuffer Exchanger 94 column (Pharmacia, 1·2 × 20·0 cm, 23 ml bed volume). Elution with 200 ml of Polybuffer 74–HCl, pH 4·0, at 35 ml h^{-1} resulted in a pH gradient from pH 7·0 to pH 4·0. The eluent was monitored at 280 nm and fractions of 1·8 ml were collected. Fractions containing proteins were analysed by PAGE and those containing P1 were combined. Polybuffer was separated from the protein by repeated gel filtration on a Sephadex G-75 column (1·3 × 80·0 cm, 70 ml bed volume), pre-equilibrated with water, at a flow rate of 4 ml h^{-1}.

Antiserum preparation

About 140 µg of purified P1 was dissolved in 100 µl of water and applied to about 2 cm^2 of nitrocellulose. The nitrocellulose was pulverized in liquid nitrogen in a mortar and suspended in 2·65 ml of phosphate buffered saline (PBS) and an equal volume of

Freund's incomplete adjuvant. Initially, one-quarter of the mixture was injected into a rabbit at many subcutaneous sites, and 2 weeks later the remaining three quarters of the mixture were injected. The rabbit was bled 2, 4, 6 and 8 weeks after the last injection. The blood samples were kept at room temperature for 3 h, to allow clotting to occur, and then stored overnight at 5 °C before centrifugation at 2500 ***g*** for 10 min. The supernatant was used as an antiserum for the detection of proteins on western blots. Antiserum raised against apoplastic fluid originating from the interaction *Cf5*/race 5 was the same as used by de Wit *et al.* [*6*].

Polyacrylamide gel electrophoresis (PAGE)

PAGE under low pH, non-denaturing conditions on 10% (w/v) polyacrylamide slab gels with pyronine Y as a front marker was performed using a method described by Reisfeld *et al.* [*23*]. A staining and fixing method was used which detects basic and low molecular weight peptides [*24*].

Sodium dodecyl sulphate–PAGE on 15% (w/v) polyacrylamide slab gels was performed as described by de Wit *et al.* [*10*], using a separation time of about 6 h. Proteins in apoplastic fluids were precipitated overnight with 80% (v/v) acetone, then suspended in water and any insoluble substances were removed by centrifugation at 16 000 ***g*** for 5 min. The residue of the supernatant obtained after freeze-drying was dissolved in 30 µl of sample buffer [0·05 M Tris–HCl, pH 6·8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercapto ethanol and 0·0025% (w/v) bromophenol blue], heated for 3 min in a boiling water bath and applied to the gel. Gels were stained in a solution of methanol:acetic acid: water (40:10:50, v/v/v) and 0·1% (w/v) Coomassie brilliant blue R-250 and destained in a solution containing methanol:acetic acid:water (10·0:7·5:82·5, v/v/v). For an estimation of the molecular weight of protein P1, markers ranging from 14·2 to 66 kD (Sigma) were used.

Electrophoretic transfer of proteins (western blotting) and radioimmunological detection

A method originally described by Towbin *et al.* [*25*] and modified by de Wit *et al.* [*6*] was used to transfer the proteins to a nitrocellulose membrane electrophorectically, for the immunoblot assay with rabbit antiserum and the radioimmunological detection of the antigen-antibody complexes.

RESULTS

Purification of the protein (P1) specific for compatible C. fulvum*–tomato interactions*

Low pH, non-denaturing PAGE of apoplastic fluids originating from various interactions between *C. fulvum* and tomato, obtained 14 days after inoculation, resulted in the protein profiles presented in Fig. 1. At this stage of the infection process, protein bands 2, 3, 4, 5 and 6 were present in fluids from both compatible (lanes c and d) and incompatible interactions (lane b), while protein bands 1 and 7 were only present in fluids from compatible interactions [*12*]. Protein band 7, the necrosis inducing peptide, represents the putative product of avirulence gene A9, which is not produced by race 2.4.5.9 [*12*] and would therefore not be present in apoplastic fluids originating from the interaction Cf5/race 2.4.5.9 (lane d). Protein band 1 (P1) was found in fluids from all compatible interactions studied and could therefore represent a factor important for basic compatibility between tomato and *C. fulvum* [*6*].

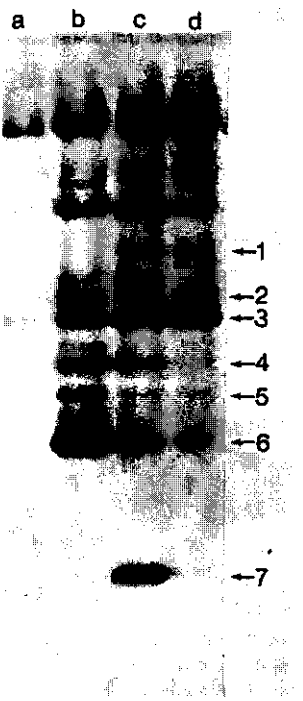

FIG. 1. Polyacrylamide gel electrophoresis profiles of apoplastic fluids obtained 14 days after inoculation. The proteins present in 300 µl of apoplastic fluid were separated under low pH, non-denaturing conditions and stained with Coomassie brilliant blue R-250. a, Control plant Cf5; b, incompatible interaction (Cf4/race 5); c, compatible interaction (Cf5/race 5); d, compatible interaction (Cf5/race 2.4.5.9). Note that protein 1 (P1) is present only in the apoplastic fluids from the two compatible interactions.

Freeze-dried apoplastic fluid originating from the compatible interaction, Cf4/race 4, obtained 14 days after inoculation, was dissolved in 25 mM sodium acetate buffer, pH 4·5, and applied to a CM-Sephadex C-25 column. Proteins 1–7 bound to the column and the NaCl gradient resulted in differential elution of the proteins. The proteins showing lower electrophoretic mobility than P1 on low pH polyacrylamide gels were for the greater part eluted at the start of the NaCl gradient, followed by P1 in combination with proteins 2–7 at higher NaCl concentrations of the eluent (Fig. 2, lane b).

Applying the combined fractions from the CM-Sephadex C-25 column which contained P1 to a Polybuffer Exchanger 94 column, resulted in elution of protein P1 within the pH gradient between pH 5·5 and 5·1 (Fig. 2, lane d). Proteins 2, 4, 5, 6 and 7 did not bind to the column at pH 7·4 and were eluted in the void volume (Fig. 2, lane c).

Molecular mass estimation of protein P1

Purified P1 migrated as one band on SDS-polyacrylamide gels and the molecular mass was estimated to be 14 kD (Fig. 3, lane c). In addition to P1, there were other marked

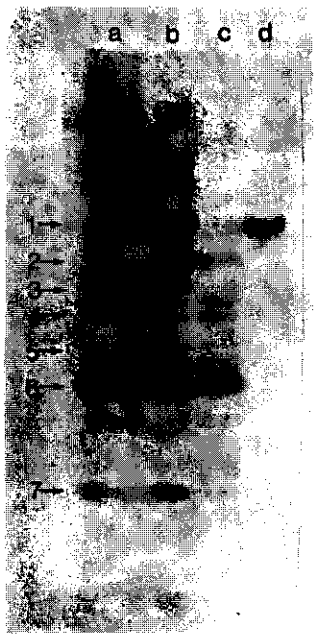

FIG. 2. Low pH polyacrylamide gel electrophoresis protein profiles of fractions obtained at different stages of purification of P1 from the apoplastic fluid. The proteins were separated under low pH, non-denaturing conditions and stained with Coomassie brilliant blue R-250. a, Untreated apoplastic fluid from a compatible interaction (Cf4/race 4); b, the combined fractions containing P1 in combination with proteins 2 to 7, collected by elution of the CM-Sephadex column with a NaCl gradient; c, fractions containing proteins which did not bind to the Polybuffer Exchanger 94 column; d, fractions collected within the pH gradient, containing P1.

differences in proteins below 20 kD between the protein profiles of apoplastic fluids originating from an incompatible interaction (Cf4/race 5; lane a) and a compatible interaction (Cf5/race 5; lane b) (Fig. 3).

Specificity of antiserum raised against P1

Antiserum raised against complete apoplastic fluid obtained from the interaction Cf5/race 5, 14 days after inoculation, did not interact with P1, indicating that antiserum raised against total apoplastic fluid does not contain antibodies against P1. This antiserum does, however, contain antibodies to the PR-like proteins 2, 3 and 5 [*6*] (results not shown). Complete apoplastic fluid appears to contain so many antigenic proteins that no, or very few, antibodies are produced against the presumably weekly antigenic protein P1. However, antibodies were indeed produced as evidenced when blots were incubated with antiserum produced from rabbits injected with purified P1 (Fig. 4, a replica of the gel presented in Fig. 1). P1 was present only in apoplastic fluids from compatible *C. fulvum*–tomato interactions (lanes c and d) since no interactions between antibodies and proteins occurred with fluids from control plants or incompatible interactions (lanes a and b).

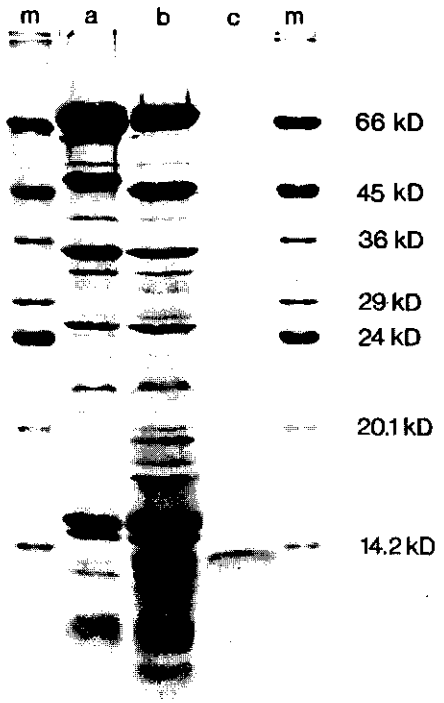

FIG. 3. SDS-polyacrylamide gel electrophoresis profiles of apoplastic fluids obtained 14 days after inoculation from: a, an incompatible interaction (Cf4/race 5); b, a compatible interaction (Cf5/race 5); c, purified P1. Lanes a and b represent proteins present in 200 µl of apoplastic fluid and lane c represents about 5 µg of P1. The two outer lanes, m, contain molecular weight markers. Proteins were stained with Coomassie brilliant blue R-250.

Time course of accumulation of P1

In a time-course experiment apoplastic fluids from the compatible interactions Cf4/race 4 and Cf5/race 5 were recovered 4, 6, 8, 10, 12 and 14 days after inoculation with conidia of *C. fulvum*. In both interactions protein P1 could be detected on nitrocellulose blots of SDS-polyacrylamide gels of the apoplastic fluids, 8 days after inoculation with the fungus. At this stage of infection in compatible interactions, fungal growth is significantly more advanced than in incompatible interactions [*10*]. The results of the interaction Cf4/race 4 are presented in Fig. 5A and 5B.

Origin of protein P1

In an attempt to determine if protein P1 is a product of the host or the pathogen, soluble proteins in the apoplastic fluids from different sources were examined as follows: (a) different *C. fulvum*–tomato interactions, (b) tomato inoculated with *P. infestans* (tomato strain), (c) healthy tomato plants, and (d) mycelial homogenates of *C. fulvum* grown *in*

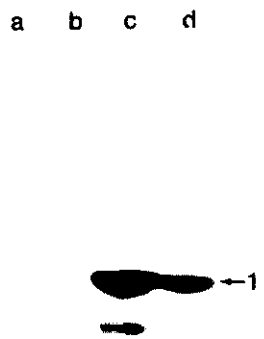

FIG. 4. Autoradiographic immunoassay performed on a nitrocellulose blot of a gel similar to that presented in Fig. 1, except that half the amounts of protein were applied. The blot was incubated with antiserum raised against purified P1 and the antibody-protein complexes were detected by ^{125}I-labelled protein A. See Fig. 1 for details of lanes.

vitro. The results are presented in Figs 6, 7A and 7B. Two replicates of the SDS-polyacrylamide gel as presented in Fig. 6 were blotted onto nitrocellulose. One blot was incubated with antiserum raised against total apoplastic fluid of the interaction Cf5/race 5 while the other was incubated with antiserum raised against purified P1.

Most antibodies present in antiserum raised against the total apoplastic fluid of the compatible interaction Cf5/race 5 bound to proteins which were also present in apoplastic fluids from incompatible interactions or healthy controls (Fig. 7A). Only one protein (other than P1) was unique to the compatible interaction (arrowed in Fig. 7A). From the results obtained with antiserum raised against the purified P1 (Fig. 7B) it can be concluded that P1 is neither present in mycelium of *C. fulvum* grown *in vitro* (lane f), nor in the apoplastic fluids from healthy tomato leaves (lane a). More importantly, P1 was not present in apoplastic fluids from incompatible *C. fulvum*–tomato interactions (lane b) nor in compatible *P. infestans*–tomato interactions (lane e). Indeed P1 was only present in compatible *C. fulvum*–tomato interactions (lanes c and d).

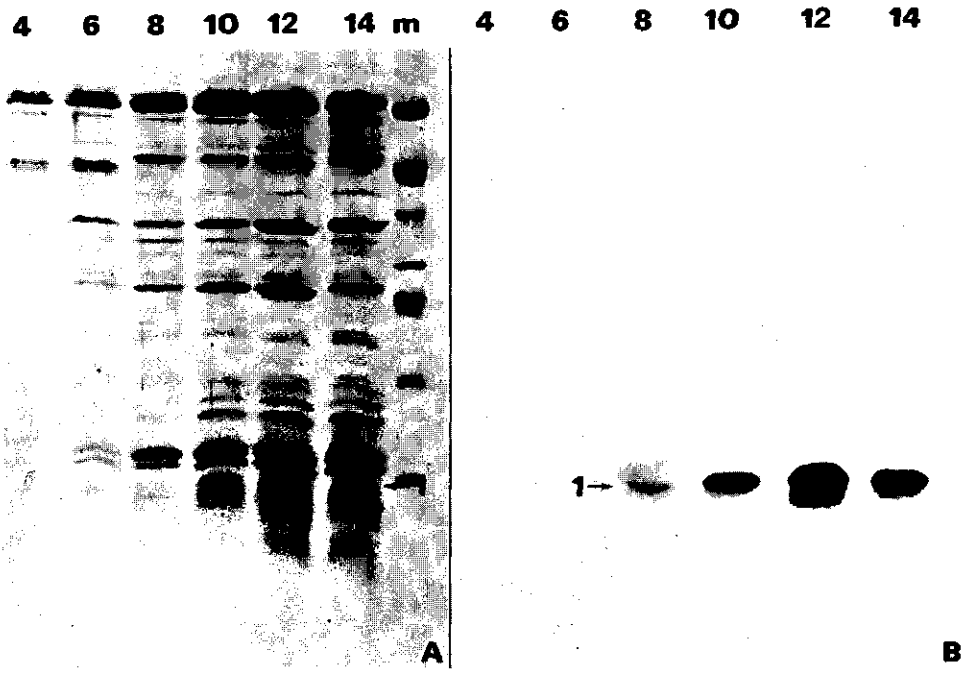

FIG. 5. Time-course of accumulation of protein P1 in apoplastic fluids from the compatible interaction Cf4/race 4. A, SDS-PAGE of apoplastic fluids recovered 4, 6, 8, 10, 12 and 14 days after inoculation. Lane m contains molecular markers. B, Radio immunological detection of antibody-protein complexes by ^{125}I-labelled protein A after incubation of a blot of a replica of the gel presented in A, with antiserum raised against P1. Note that protein P1 was first detected eight days after inoculation.

In order to determine if P1 accumulates in compatible *C. fulvum*–tomato interactions as a result of *de novo* synthesis, or if it is constitutively present in the host and released by a fungus-mediated process, homogenates of non-inoculated, incompletely expanded upper leaves, of older leaves and of inoculated leaves were electrophoresed under low pH, non-denaturing conditions and blotted onto a nitrocellulose membrane. P1 was only detected in leaf homogenates of compatible *C. fulvum*–tomato interactions (results not shown), indicating that the protein is not released by a fungus-mediated process or by fungal imposed stress on plant cells.

DISCUSSION

Ion-exchange chromatography followed by chromatofocusing proved to be a useful method for the purification of protein P1 to homogeneity.

Injection of complete apoplastic fluids from compatible *C. fulvum*–tomato interactions containing protein P1 into rabbits did not result in the production of antibodies against this protein and antibodies to protein P1 were only obtained when purified P1 was used as the antigen. Apparently injection of rabbits with a mixture of proteins and glycoproteins leads mainly to antibodies directed against the most antigenic components

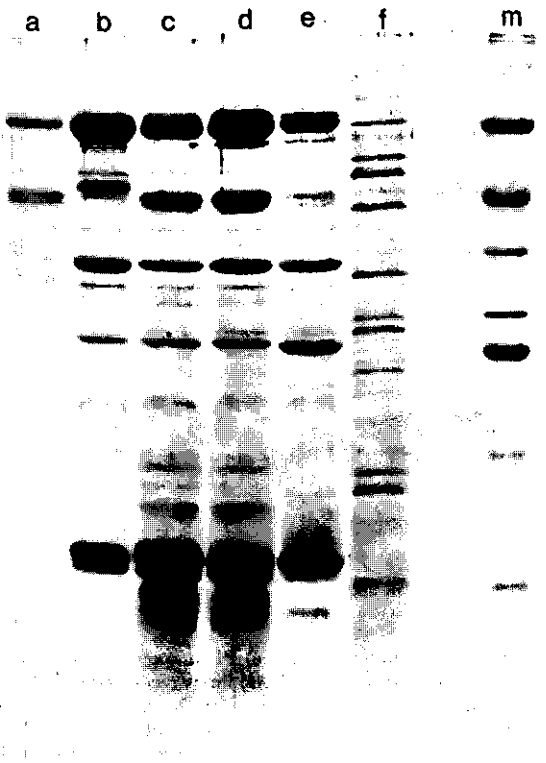

FIG. 6. Polyacrylamide gel electrophoresis profiles of proteins separated under SDS-denaturing conditions and stained with Coomassie brilliant blue R-250. Lanes a–e represent 200 µl of apoplastic fluid, originating from: a, a non-inoculated control plant (Cf5); b, incompatible interaction (Cf4/race 5); c, compatible interaction (Cf5/race 5); d, compatible interaction (Cf5/race 2.4.5.9); e, compatible interaction between *Phytophthora infestans* and tomato, 6 days after inoculation. Apoplastic fluids of the interactions between *Cladosporium fulvum* and tomato were obtained 14 days after inoculation. Lane f represents the protein profile obtained from a homogenate of 50 mg of mycelium of race 5, grown in culture. Lane m contains molecular mass markers. See Fig. 3 for details.

and antibodies to weakly antigenic proteins can only be obtained using purified proteins as antigens.

The unique occurrence of protein P1 in compatible *C. fulvum*–tomato interactions raises questions concerning its origin and function. As no other type of plant stress or fungal infection other than *C. fulvum* tested by us led to the accumulation of protein P1, it is clearly unrelated to the class of proteins known as pathogenesis-related (PR) proteins [*3, 4, 6, 13, 14, 21, 22, 27*]. PR proteins are of plant origin and can be induced by many types of stress, including fungal and viral infections. Although protein P1 and the PR protein P14 have a similar molecular mass of 14 kD [*3, 4, 10, 21, 22*], they are totally unrelated.

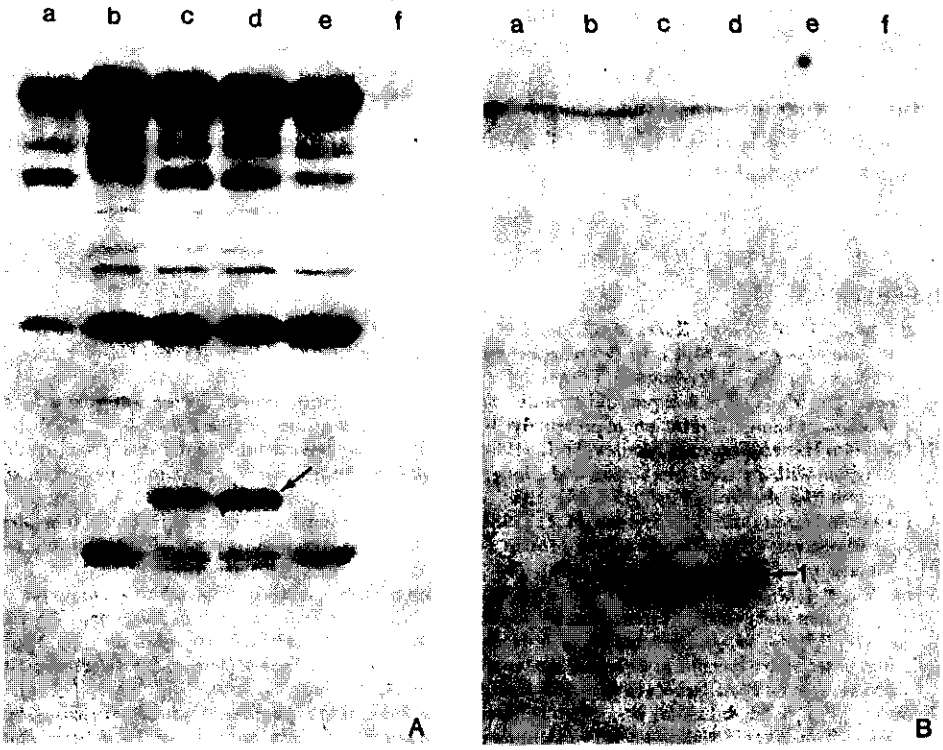

FIG. 7. Autoradiographic immunoassay performed on nitrocellulose blots of gels similar to the one presented in Fig. 6. The blot was incubated with antiserum raised against apoplastic fluid from the compatible interaction Cf5/race 5 (A), and antiserum raised against the purified P1 (B) and the antibody–protein complexes were detected by ^{125}I-labelled protein A. The amount of proteins applied to the gels in Fig. 7A and B was half that used in Fig. 6. The arrow in part A indicates the protein detectable only in the two compatible interactions.

Protein P1 could be of fungal origin, but antibodies raised against it did not interact with soluble proteins extracted from *C. fulvum* grown *in vitro* in liquid shake culture, or on solid agar, implying that if it is of fungal origin, it is only produced *in vivo* during the infection process. Specific synthesis of protein P1 during infection suggests that it might be an important factor of basic compatibility [*16, 18*]. As the production of protein P1 is not limited to specific compatible race–cultivar interactions it is probably not involved in virulence of *C. fulvum* directed against specific tomato cultivars.

The possibility still remains that protein P1 is a protein of host origin, which specifically accumulates after infection of tomato by *C. fulvum*. Protein P1 may accumulate specifically in tomato leaves after infection by *C. fulvum*, analogous to nodulins which are plant proteins of which the encoding genes are specifically expressed in legumes after infection by *Rhizobium* spp. [*26*]. Leguminous root nodules contain at least 20 different nodulins which are expressed at different stages of nodule formation [*15*]. Preliminary experiments on the localization of protein P1 in the infected leaf, however, indicated that it is only present in and around the cell walls of the hyphae of *C. fulvum* and not in the cell walls of the host.

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CHAPTER 3

CARBOHYDRATE COMPOSITION OF APOPLASTIC FLUIDS ISOLATED FROM TOMATO LEAVES INOCULATED WITH VIRULENT OR AVIRULENT RACES OF *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)

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Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of Cladosporium fulvum (syn. Fulvia fulva)[1]

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Abstract

Inoculation of tomato (*Lycopersicon esculentum*) with virulent races of *Cladosporium fulvum* (compatible interactions), resulted in substantial changes of the carbohydrate composition of apoplastic fluids isolated from the leaves, during the course of the infection process. In addition to a decrease in the concentration of the translocation sugar sucrose, a transient accumulation of the hexoses glucose and fructose and an accumulation of the polyol mannitol were observed. The latter coincided with a rising level of mannitol dehydrogenase, an enzyme that reduces fructose to mannitol. Only minor changes were detected in the carbohydrate composition of apoplastic fluids isolated from leaves of uninoculated control plants or plants inoculated with avirulent races of *C. fulvum* (incompatible interactions). The fungal metabolite mannitol was not detected in apoplastic fluids isolated from the latter plants.

These results suggest that, upon colonization of the intercellular spaces by virulent races of *C. fulvum*, apoplastic sucrose is hydrolyzed by a host and/or fungal invertase and the resulting hexoses, glucose and fructose, are converted into mannitol by the fungus. In incompatible tomato-*C. fulvum* interactions a functional nutritional relationship between plant and fungus is prevented by plant defense responses, which might explain why in these interactions the carbohydrate composition of apoplastic fluids is similar to that of uninoculated control plants.

Additional keywords: Lycopersicon esculentum, sucrose, glucose, fructose, mannitol, mannitol dehydrogenase, invertase.

Introduction

Cladosporium fulvum (Cooke) (syn. *Fulvia fulva* [Cooke] Cif.), the causal agent of tomato leaf mould, is a biotroph that colonizes the intercellular spaces between mesophyll cells of leaves of tomato (*Lycopersicon esculentum* Mill.), without the formation of haustoria (Lazarovits and Higgins, 1976a, b; De Wit, 1977).

In a compatible tomato-*C. fulvum* interaction, where extensive fungal colonization takes place, the intercellular hyphae are closely appressed to the host cell walls, usually causing slight indentation of the walls. Relatively few ultrastructural changes of the

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host mesophyll cells occur until the fungus starts to sporulate, indicating that initially a stable biotrophic relationship exists between host and parasite. In the incompatible interaction a rapid defense response by host cells (e.g. callose deposition, accumulation of phytoalexins, production of pathogenesis- related (PR) proteins) prevents the establishment of a functional nutritional relationship between host and pathogen (Lazarovits and Higgins, 1976b).

Up to now little is known about the mechanism by which this fungus obtains the carbohydrates necessary for growth and reproduction. A candidate is sucrose, the main product of photosynthesis, which is either exported into the phloem, temporarily stored in the leaf, or metabolized. Although the mechanism by which sucrose is transported from mesophyll cells into the phloem of tomato leaves is unknown, there exists substantial support for a loading of the phloem via the apoplast (Giaquinta, 1983). Apoplastic sucrose could serve as an important carbon source for *C. fulvum*, as this fungus does not produce cell wall degrading enzymes to release carbon-containing compounds from plant cell walls.

Studies by Lewis and Harley (1965) showed that the mycorrhizal fungus of beech is able to absorb carbohydrates from the host and to convert them into mannitol, trehalose and glycogen. As mannitol is the most abundant reserve sugar in this mycorrhizal fungus and the plant is unable to utilize this polyol, the hypothesis was put forward that the fungus absorbs carbohydrates from the host and transforms them into sugars that can only be metabolized by the fungus. In this way a concentration gradient with respect to the carbohydrates of the host is created. As many plants cannot metabolize polyols, Lewis and Harley (1965) suggested that this hypothesis may not only hold for mycorrhizal fungi but also for fungi pathogenic on plants. In their review Smith and coworkers (1969) suggested that biotrophic parasites such as rusts, smuts and powdery mildews obtain carbohydrates from their hosts in a way similar to that of the mutualistic ectotrophic mycorrhizal fungi. Sucrose, the common translocation sugar, was shown to be the main carbohydrate source for these parasitic fungi. It was hydrolyzed to glucose and fructose, while subsequently the latter two compounds were taken up and converted into fungal carbohydrates such as trehalose, mannitol and arabitol. There are several reports on the redirection of the translocation of the assimilatory carbohydrates of the host upon infection by biotrophic or hemibiotrophic fungi (Long and Cooke, 1974; Long et al., 1975; Billett et al., 1977; Mitchell et al., 1978; Clancy and Coffey, 1980; Callow et al., 1980; Brem et al., 1986; Hwang and Heitefuss, 1986; Hwang et al., 1989). In many cases an increased invertase activity (from host and/or fungus) was responsible for sucrose depletion and increases in the concentration of the hexoses glucose and fructose, while many carbohydrates of fungal origin could be detected in the infected tissue.

The present study was undertaken to investigate whether the described mechanism of nutrient uptake described above also holds for *C. fulvum* and to what extent plant defense mechanisms are affecting the nutritional relationship between plant and fungus. In contrast to previous studies, where the sugars present in total leaf extracts were examined, this study focuses on changes in carbohydrates present in the apoplast. The changes in carbohydrate composition of apoplastic fluids in relation to the mode of nutrition of the intercellularly growing fungus are discussed, as well as the possibility of using mannitol concentration or mannitol dehydrogenase activity present in apoplastic fluids as a way of estimating fungal biomass in infected tomato leaves.

Materials and methods

Plant, fungus and inoculation. Cultivation of tomato plants and races of *C. fulvum* and inoculation were carried out as described previously (De Wit, 1977; De Wit and Flach, 1979). Conidial suspensions of races 4 and 5 of *C. fulvum* were used to inoculate two near-isogenic lines of tomato, carrying resistance gene Cf4 (susceptible to race 4 and resistant to race 5) or Cf5 (susceptible to race 5 and resistant to race 4). Uninoculated control plants were kept under the same conditions as the inoculated ones.

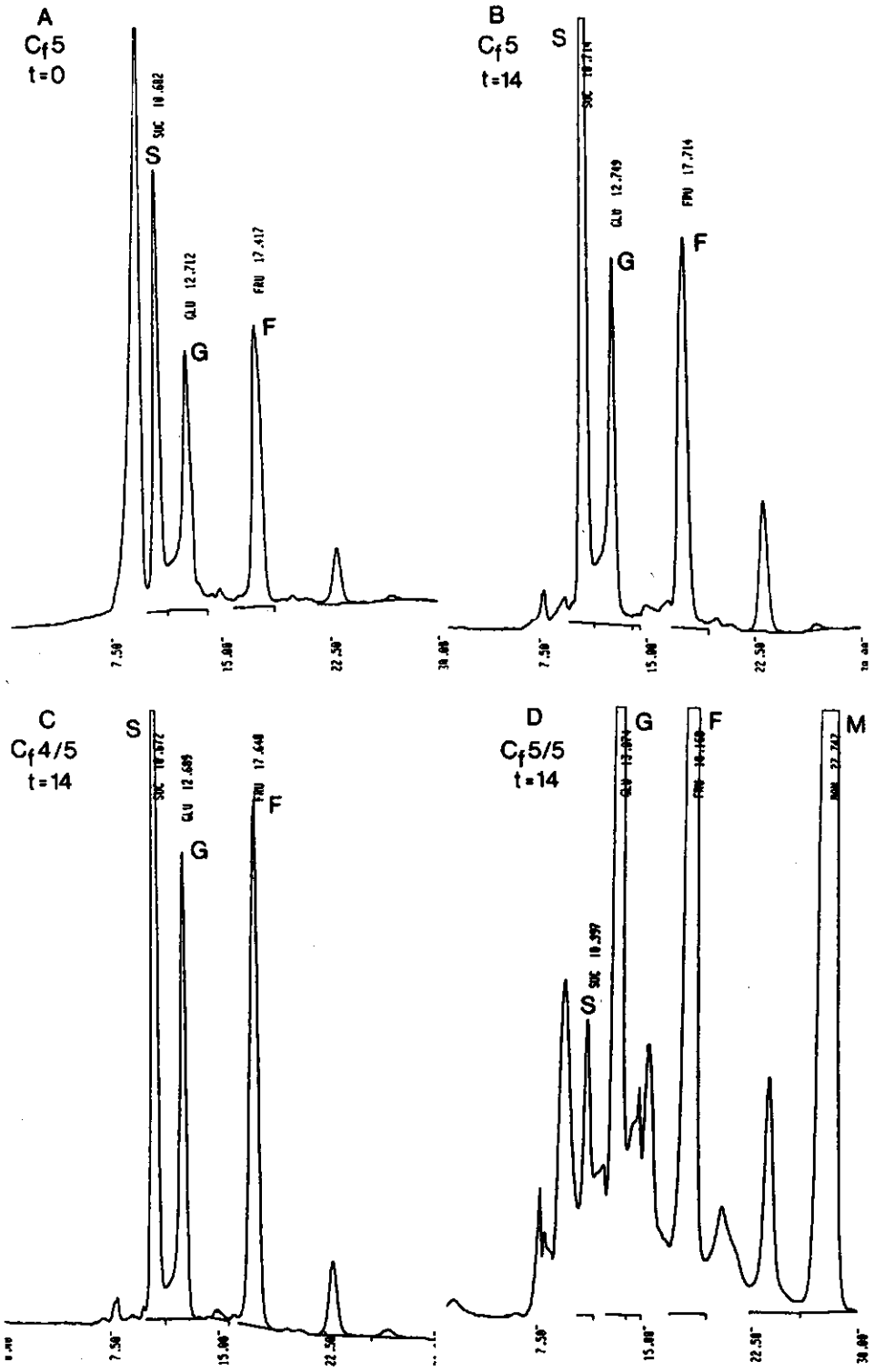
Preparation of apoplastic fluids and analysis of carbohydrate composition. For time-course experiments about 15 to 25 plants were inoculated for every interaction and 10 to 20 leaflets were picked at random at 3, 5, 7, 9, 12, and 14 days after inoculation. The whole leaflets were infiltrated with distilled water *in vacuo* and apoplastic fluid was isolated by centrifugation, according to the method described by De Wit and Spikman (1982).

Proteins present in 300-μl aliquots of apoplastic fluid were precipitated overnight at -20 °C with four volumes of acetone and pelleted by centrifugation at 1500 *g* (5 min). The acetone was evaporated from the supernatant and the remaining aqueous solution was freeze-dried. The residue was dissolved in 100 μl of H_2O, centrifuged at 15 000 *g* (5 min) and analyzed by high pressure liquid chromatography (HPLC). Twenty μl was injected onto an Aminex Carbohydrate HPX-87P column (Bio-Rad, 300 $\times$ 7.5 mm, at 85 °C) fitted into a Waters HPLC system. The column was eluted with H_2O at a flow rate of 0.6 ml min^{-1} and the eluate was monitored using a refractive index detector. The amounts of glucose, fructose, sucrose and mannitol were calculated using the respective sugars as external standards. Other external standards used were xylose, galactose, arabinose and inositol.

Mannitol dehydrogenase (MTLDH) assay. MTLDH activity in apoplastic fluids was assayed by monitoring oxidation of NADPH at 340 nm in the presence of fructose (Morton et al., 1985). In a total volume of 1 ml, a sample of untreated apoplastic fluid (50 to 100 μl) was incubated in 50 mM Tris-HCl buffer, pH 7.5, containing 800 mM fructose and 0.25 mM NADPH. In this assay one unit of activity represents the amount of enzyme which catalyses the oxidation of 1 nmole NADPH min^{-1}.

Results

Sugar composition of apoplastic fluids isolated from uninoculated and inoculated tomato leaves. To investigate the mechanism of nutrient uptake for *C. fulvum* several time-course experiments were carried out. Here we describe the results of a representative experiment using uninoculated Cf5 plants, the incompatible interaction Cf4/race 5 and the compatible interaction Cf5/race 5. The chromatogram presented in Fig. 1A shows that sucrose, glucose and fructose are the main sugars present in apoplastic fluid obtained from uninoculated Cf5 plants that were kept under greenhouse conditions. One ml of apoplastic fluid contained about 150 μg of each of the three sugars. The compound eluting around 23.0 min was identified as inositol and the peak preceeding sucrose contained an unidentified substance. This peak may consist of a mixture of substances (phenols, oligosaccharides) which were not separated on the column.


A
Cf5
t=0
S
G
F
B
Cf5
t=14
S
G
F
C
Cf4/5
t=14
S
G
F
D
Cf5/5
t=14
G
F
M
S

◀ Fig. 1. HPLC analysis of the mono- and disaccharides present in apoplastic fluids. A and B show the HPLC traces of apoplastic fluids obtained from leaves of uninoculated Cf5 plants before and after incubation under the same conditions as the inoculated plants for 14 days, respectively. In C and D the analysis of apoplastic fluids (isolated 14 days after inoculation) from the incompatible interaction Cf4/race 5 and the compatible interaction Cf5/race 5 is shown. The peaks representing sucrose, glucose, fructose and mannitol are marked S, G, F and M, respectively.

In the uninoculated Cf5 plants, kept under the same conditions as the inoculated ones for 14 days, the apoplastic sugar concentrations had changed (Fig. 1B). There was an increase in sucrose and fructose levels and the peak of unidentified substances had disappeared after 14 days. Apoplastic fluid isolated from the incompatible Cf4/race 5 interaction showed about the same sugar content as the uninoculated control plants that had been incubated under the same conditions (Fig. 1C). In the compatible Cf5/race 5 interaction substantial changes in apoplastic sugar composition had occurred at 14 days after inoculation (Fig. 1D). At this stage the leaf tissue was heavily colonized by the fungus. Besides a decrease in sucrose concentration, levels of glucose and fructose had increased 4- to 14-fold, respectively. The compound with a retention time around 27.7 min was identified as mannitol and reached a concentration of more than 2000 $\mu g\ ml^{-1}$ of apoplastic fluid. There were no changes in the inositol content of the apoplastic fluids isolated from the uninoculated or inoculated plants within 14 days after inoculation. In addition to the five sugars discussed above, several minor peaks were detected but could not be identified with the set of external sugar standards used.

Changes in carbohydrate composition of apoplastic fluids during the infection process. Time-course experiments showed that in apoplastic fluids isolated from leaves of uninoculated Cf5 plants or Cf4 plants inoculated with race 5 of *C. fulvum* (incompatible interaction), an increase in sucrose, glucose and fructose concentration occurred upon incubation under conditions of high relative humidity. However, compared to the apoplastic fluid isolated from the compatible interaction Cf5/race 5, the glucose and fructose levels remained low (Fig. 2A,B). In the compatible interaction, a twofold increase in sucrose content during the first 7 days was followed by a decrease in sucrose content which coincided with a substantial increase in glucose, fructose and mannitol concentrations (Fig. 2C). From 9 days after inoculation onwards the sucrose level decreased slowly, but a sharp decrease of glucose and fructose was observed, while mannitol accumulation slowly reached its maximum in this period.

In the compatible Cf5/race 5 interaction, when using a low inoculum concentration, fungal biomass in the intercellular spaces remained low, even when abundant sporulation was visible at 10 to 12 days after inoculation. There was no visual damage to the leaves up to 18 days after inoculation. Although the amounts of the different sugars remained at a much lower level compared to the Cf5/race 5 interaction where colonization was abundant as a result of using a high inoculum concentration and no sporulation was visible, the same trends in sugar accumulation could be observed (results not shown). A decrease in sucrose content coincided with a fast increase in glucose, fructose and mannitol, followed by decreases in glucose and fructose levels when sucrose concentration was low. At 18 days after inoculation the mannitol content had reached 600 $\mu g\ ml^{-1}$ of apoplastic fluid.

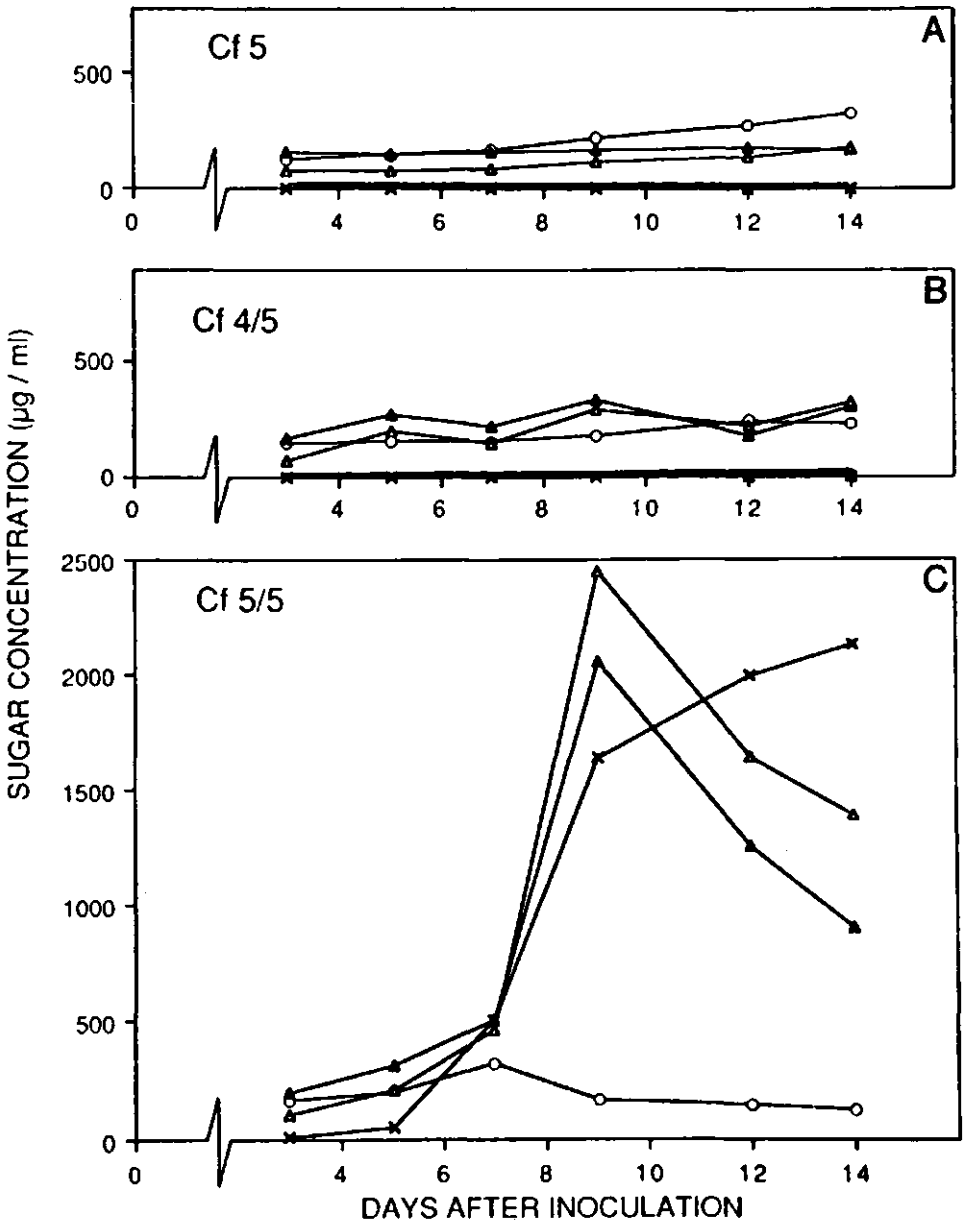


Fig. 2. Time-course of sucrose (○), glucose (▲), fructose (△) and mannitol (×) concentration in apoplastic fluids. Apoplastic fluids were isolated from leaves of uninoculated Cf5 plants (A) and from leaves of tomato cultivars Cf4 (B) and Cf5 (C) inoculated with race 5 of *C. fulvum*. Apoplastic fluids were isolated 3, 5, 7, 9, 12 and 14 days after inoculation and the carbohydrate composition was determined as described in materials and methods.

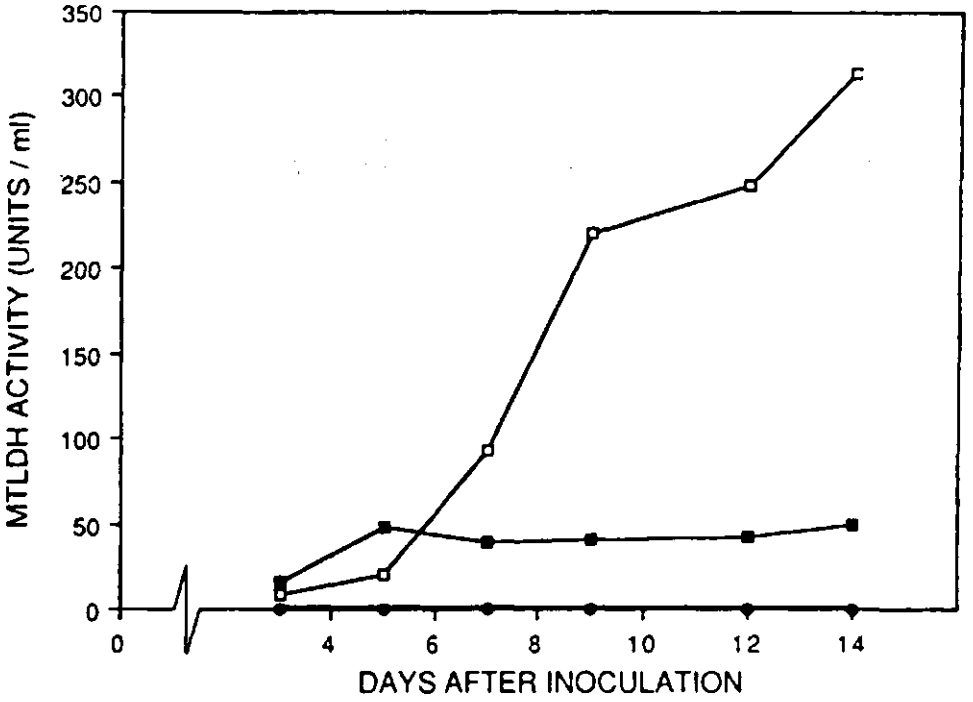


Fig. 3. Time-course of mannitol dehydrogenase (MTLDH) activity in apoplastic fluids. Apoplastic fluids were isolated from leaves of tomato cultivars Cf4 (■) and Cf5 (□) inoculated with race 5 of *C. fulvum* and from leaves of uninoculated Cf5 plants (●). Apoplastic fluids were obtained 3, 5, 7, 9, 12 and 14 days after inoculation and MTLDH activity was determined as described in materials and methods.

Similar results were obtained in time-course experiments with the incompatible interaction Cf5/race 4 and the compatible interaction Cf4/race 4. However, in general race 4 did grow less abundantly than race 5, resulting in a lower rate of accumulation of the various carbohydrates in the Cf4/race 4 interaction (results not shown).

Changes in mannitol dehydrogenase activity present in apoplastic fluids during the infection process. Measurements of apoplastic mannitol dehydrogenase (MTLDH) activity are presented in Fig. 3. MTLDH converts fructose into mannitol, using NADPH as a cofactor. In apoplastic fluid obtained from the leaves of uninoculated Cf5 plants no MTLDH activity could be detected, while in the incompatible Cf4/race 5 interaction a fast increase was observed within the first 5 days. In the compatible Cf5/race 5 interaction, a slower initial increase was followed by a substantial rise in MTLDH activity between day 5 and 9 after inoculation. No NADPH oxidation could be detected in control assays where fructose or apoplastic fluid was omitted.

Measurements on MTLDH activity in apoplastic fluids isolated from the interactions with race 4 gave similar results, but the differences were less pronounced (results not shown).

Discussion

Changes in the metabolism of host carbohydrates (sucrose, glucose and fructose) after infection by biotrophic fungi have been frequently reported (Long and Cooke, 1974; Long et al., 1975; Billett et al., 1977; Mitchell et al., 1978; Clancy and Coffey, 1980; Callow et al., 1980; Brem et al., 1986; Hwang and Heitefuss, 1986; Hwang et al., 1989). In most cases increased invertase activity of host and/or fungus resulted in a depletion of sucrose and the production of high levels of the hexoses glucose and fructose. Besides these quantitative changes in host sugars, mannitol, trehalose and/or erythritol were detected as fungal metabolites.

In *C. fulvum*-infected tomato leaves similar changes in the carbohydrate composition of apoplastic fluids were observed. During the colonization process of the intercellular spaces of tomato leaves a sucrose depletion, transient accumulation of glucose and fructose and accumulation of the polyol mannitol were observed. Possibly the fungus induces leakage of sucrose into the apoplast by changing the permeability of the plasma membrane, as a substantial rise in apoplastic sucrose concentration was observed during the early stages of infection in the compatible Cf5/race 5 interaction. The drop in the amount of sucrose between day 7 and 9 after inoculation was correlated with an increase of glucose and fructose in the apoplast, suggesting that these substances accumulate at the cost of the apoplastic sucrose. At the same time the concentration of mannitol increased substantially. The drop in glucose and fructose levels around 9 days after inoculation coincided with a levelling-off of the mannitol accumulation, suggesting that this accumulation is dependent on glucose and fructose.

These results suggest that apoplastic sucrose is hydrolyzed by host and/or fungal invertases and the resulting hexoses, glucose and fructose, are metabolized by the fungus. Glucose can be converted into fructose, which is subsequently converted into mannitol by MTLDH. Presumably hydrolysis of sucrose occurs in the apoplast and the generated glucose and fructose are absorbed by the fungus and subsequently metabolized into mannitol. Part of the mannitol may either be actively exported or leach passively into the apoplast. In incompatible tomato-*C. fulvum* interactions plant defense responses prevent the establishment of a functional nutritional relationship, resulting in apoplastic sugar compositions similar to those of uninoculated plants.

The relatively high activity of MTLDH measured initially in apoplastic fluid isolated from the incompatible Cf4/race 5 interaction could be the result of a release of MTLDH from the penetrating hyphae caused by deleterious effects of plant defense responses. These effects could be partly caused by the hydrolytic activity of apoplastic 1,3-β-glucanases and chitinases which are rapidly induced in the incompatible combination (Joosten and De Wit, 1989). In the compatible combination the course of MTLDH activity followed that of mannitol accumulation.

When heavy sporulation takes place the amounts of the various sugars detected remain at a lower level. This may be because intercellular growth is less extensive and storage carbohydrates such as mannitol are metabolized to provide energy for spore production or are directly translocated to the spores. Mannitol is the most common polyol in fungi (Lewis and Smith, 1967) and could serve as a carbohydrate reserve or a translocatory compound. It could also have a function in osmoregulation, storage of reducing power and coenzyme regulation (Jennings, 1984). As mannitol could not be detected in uninoculated control plants or incompatible interactions, its presence

in apoplastic fluids can be used as a marker for compatibility. Among the various methods to estimate the amount of fungal biomass present in plant tissue (Matcham et al., 1984) the accumulation of the polyol could be a reliable alternative.

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Samenvatting

Koolhydraatsamenstelling van apoplastische vloeistoffen geïsoleerd uit tomatebladeren geïnoculeerd met virulente of avirulente fysio's van Cladosporium fulvum (syn. Fulvia fulva)

Inoculatie van tomaat (*Lycopersicon esculentum*) met virulente fysio's van *C. fulvum* (compatibele interacties), leidde tot aanzienlijke veranderingen in de koolhydraatsamenstelling van apoplastische vloeistoffen die uit de bladeren werden geïsoleerd in de loop van het infectieproces. Naast een sterke daling van de concentratie van de transportsuiker saccharose, vond er ophoping van de hexoses glucose en fructose en de polyol mannitol plaats. De accumulatie van mannitol ging gepaard met een toename in de activiteit van mannitol dehydrogenase, een enzym dat fructose reduceert tot mannitol. In de koolhydraatsamenstelling van apoplastische vloeistoffen geïsoleerd uit bladeren van niet geïnoculeerde controleplanten, of planten geïnoculeerd met avirulente fysio's van *C. fulvum* (incompatibele interacties), werden slechts kleine veranderingen waargenomen. De schimmelmetaboliet mannitol kon niet worden aangetoond in de apoplastische vloeistoffen die uit deze planten werden geïsoleerd.

Deze resultaten suggereren dat bij de kolonisatie van de intercellulaire ruimtes door virulente fysio's van *C. fulvum*, saccharose uit de apoplast wordt gehydrolyseerd door invertase afkomstig van de plant of de schimmel waarna de ontstane hexoses, glucose en fructose, door de schimmel worden omgezet in mannitol. Bij incompatibele tomaat-*C. fulvum* interacties wordt een functionele voedingsrelatie tussen plant en schimmel voorkomen door het optreden van afweerreacties van de plant, hetgeen kan verklaren waarom in deze interacties de koolhydraatsamenstelling van apoplastische vloeistoffen vergelijkbaar is met die van niet geïnoculeerde controleplanten.

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CHAPTER 4

PARTIAL PURIFICATION OF THE PUTATIVE PRODUCT OF AVIRULENCE GENE *AVR4* OF *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)

Matthieu H.A.J. Joosten, Cécile J.B. Bergmans and Pierre J.G.M. De Wit

ABSTRACT

The interaction between tomato (*Lycopersicon esculentum*) and *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) is typical of a gene-for-gene relationship. This implies that the outcome of the interaction between host and fungus (compatible or incompatible) is defined by the interaction between products encoded by fungal avirulence genes, the so-called race-specific elicitors, and host resistance genes, the cultivar-specific receptors.

The putative product of avirulence gene *avr4*, the race-specific elicitor inducing necrosis in near-isogenic lines carrying resistance gene Cf4, was partially purified from apoplastic fluid that was obtained from the compatible interaction between near-isogenic line Cf5 and race 5, at three weeks after inoculation. Fractionated acetone precipitation, followed by preparative high pH gel electrophoresis and high resolution liquid chromatography (HRLC), resulted in a substantial purification of the elicitor. The putative *avr4* product precipitated in 60% (v/v) acetone, migrated on high pH, native gels and bound to an anion exchange column at pH 9.0.

In addition to the induction of necrosis or chlorosis, the elicitor preparation induced the accumulation of pathogenesis-related (PR) proteins, indicating that active host defense is triggered by recognition of the race-specific elicitor by the plant.

INTRODUCTION

The interaction between the intercellularly growing biotrophic fungal parasite *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) and tomato is assumed to be a gene-for-gene relationship (2). In this system it is supposed that host resistance is triggered by the interaction of products encoded by fungal avirulence genes with products of the corresponding resistance genes of the host. In order to elucidate the molecular basis of race-cultivar specificity, characterization of the putative products of fungal avirulence genes, the so-called race-specific elicitors, is of great importance. Following molecular characterization of these race-specific elicitors, their encoding genes and their receptors in the host, the putative products of host resistance genes, might be identified.

De Wit and Spikman (5) showed that race-specific elicitors were present in apoplastic fluids isolated from leaves of tomato cultivars inoculated with a virulent race of *C. fulvum*. The specificity of the elicitors was determined by the virulence genes present in the race of the fungus, not by the resistance genes present in the tomato cultivar (6).

One of the race-specific elicitors, a basic peptide of 28 amino acids in length, inducing a hypersensitive response (HR) in tomato cultivars carrying resistance gene Cf9, has been purified from apoplastic fluids and its amino acid sequence has been determined (7, 17). The elicitor was only present in apoplastic fluids isolated from tomato plants that were infected by races of *C. fulvum* that contained avirulence gene *avr9*; races that did not carry this avirulence gene did not produce the peptide and were virulent on Cf9 genotypes (7, 17, 18). By screening a cDNA library, made on poly(A)-RNA isolated from a compatible Cf5/race 5 interaction, with a partly degenerated oligonucleotide probe that was derived from the amino acid sequence of the elicitor, cDNA of the *avr9* gene was cloned and characterized (22). It was shown that *avr9* is a single copy gene which is absent in races virulent on tomato Cf9 genotypes. In order to obtain more support for the validity of the gene-for-gene hypothesis and to determine whether virulence of races of *C. fulvum* is generally caused by a loss of avirulence genes, race-specific elicitors other than the *avr9* product have to be purified and characterized. Here we describe the partial purification of the putative product

of avirulence gene *avr4* from apoplastic fluid isolated from the compatible interaction Cf5/race 5. The elicitor precipitated in 60% (v/v) acetone, migrated on high pH, native gels and bound to an anion exchange column at pH 9.0.

MATERIALS AND METHODS

INOCULATION AND ISOLATION OF APOPLASTIC FLUIDS

Near-isogenic line of tomato (*Lycopersicon esculentum*) Cf5 was inoculated with a conidial suspension of race 5 of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif), as described previously (3, 4). When abundant colonization of the leaves was observed (three weeks after inoculation), apoplastic fluid was isolated according to the method described by De Wit and Spikman (5). The race used in this study, its putative genotype and the outcome of its interaction with Cf4 and Cf5 tomato genotypes, are shown in Table 1.

Table I. Genotype of race 5 of *C. fulvum* and outcome of its interaction with Cf4 and Cf5 tomato genotypes. The race of *C. fulvum* is named according to its virulence gene, while tomato cultivars are named according to their resistance gene. Avirulence and resistance are supposed to be dominant (C: compatible, plant is susceptible; I: incompatible, plant is resistant).

	Race of *Cladosporium fulvum* 5
Tomato genotype	Genotype
	$A_2A_4a_5A_9$
Cf4	I
Cf5	C

PAGE

SDS-PAGE was performed as described by Joosten and De Wit (10). PAGE under high pH (pH 8.8), native conditions was performed similar to SDS-PAGE, with the modification that SDS was omitted in all buffers used. In addition, the sample buffer did not contain 2-mercapto ethanol. After separation proteins were stained with Coomassie brilliant blue R-250 (10) or silver (15).

PARTIAL PURIFICATION OF THE PUTATIVE PRODUCT OF *AVR4*.

To 250 mL of apoplastic fluid obtained from the compatible interaction Cf5/race 5, acetone was added to a final concentration of 30% (v/v) and after incubation at -20°C for two hours the precipitated proteins were pelleted by centrifugation (5 min, 1,000*g*). After adding acetone to the supernatant to a final concentration of 60% (v/v), the suspension was incubated at -20°C overnight and subsequently centrifuged at 1,000*g* for 5 min, to obtain the precipitated proteins. The 30% (v/v) and 60% (v/v) acetone pellets were air-dried, resuspended in 5 mL of H_2O, centrifuged (15 min, 13,000*g*) and the supernatants were freeze-dried. Acetone was evaporated from the 60% (v/v) acetone supernatant and the residue was resuspended in 5 mL of H_2O, centrifuged (15 min, 13,000*g*) and freeze-dried. Chlorosis- and necrosis-inducing activity of the various preparations was assessed by injection into leaflets of tomato near-isogenic line Cf4 (5, 6). Injection into leaflets of near-isogenic line Cf5 was always included as a negative control. As the majority of the race-specific elicitor activity was present in the 60% (v/v) acetone pellet, this protein preparation was used for further purification. The freeze-dried proteins were dissolved in 600 μL of loading buffer per 50 mL of original apoplastic fluid and were applied over the entire width of a 15% high pH, native polyacrylamide slab gel of 11 cm x 16 cm x 1.5 mm. After electrophoresis, 11 horizontal slices of 1 cm were cut from the gel and proteins were eluted by shaking the gel slices in two portions of 20 mL of H_2O for 24 hours at room temperature. The eluates were filtered through glasswool, freeze-dried, dissolved in 2 mL of H_2O and dialyzed against H_2O (Spectro/Por tubing; cut off 3500 D). After dialysis the volume of the eluates was adjusted to 10 mL with H_2O and they were assayed for biological activity. Chlorosis- and necrosis-inducing activity was normally assessed 2 to 4 days after injection of the leaflets.

From the eluates containing the highest biological activity 5 mL was freeze-dried, dissolved in 400 μL of 30 mM Tris-HCl buffer, pH 9.0 (buffer A) and subjected to high resolution liquid chromatography (HRLC, Bio-Rad) in two portions of 200 μL. After loading the proteins on a MA 7Q anion exchange column (Bio-Rad) that was equilibrated with buffer A, the column was washed with buffer A at a flow rate of 1.5 mL/min for 3 min and the bound proteins were subsequently eluted with a linear NaCl gradient in buffer A in two steps (0-200 mM in 15 min, followed by 200-400 mM in 4 min), at the same flow rate (fractions of 0.75 mL were collected). The absorption of the eluent was monitored at 280 nm and collected fractions were freeze-dried and pooled (in a total volume not exceeding 100 μL) according to their protein contents. After dialysis (Pierce System 100 Microdialyzer, fitted with Spectro/Por tubing, cut off 3500 D), the volume was adjusted to 500 μL with H_2O and the fractions were assayed for chlorosis- or necrosis-inducing activity by injection into leaflets of near-isogenic lines Cf4 and Cf5.

RESULTS

FRACTIONATED ACETONE PRECIPITATION OF RACE-SPECIFIC ELICITORS

Precipitation of the proteins present in apoplastic fluid obtained from the compatible interaction Cf5/race 5 by addition of acetone to a final concentration of 30% (v/v), followed by adding acetone to a final concentration of 60% (v/v), resulted in concentration of the race-specific elicitor activity in the 60% (v/v) acetone pellet. Injection of the dissolved 30% (v/v) acetone pellet or 60% (v/v) acetone supernatant revealed only a very low necrosis-inducing activity. Compared to the original apoplastic fluid, the 60% (v/v) acetone pellet contained significantly more of the smaller proteins, as can be judged by SDS-PAGE (3 kD to 20 kD, Fig. 1, compare lanes a and b).

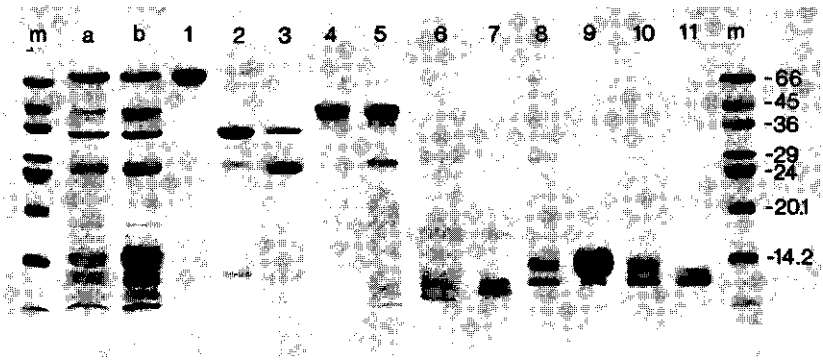

Figure 1. SDS-PAGE of the fractions obtained after fractionated acetone precipitation and native preparative high pH-PAGE of the proteins present in apoplastic fluid obtained from the interaction Cf5/race 5. Lane a contains 50 μL of apoplastic fluid, lane b contains 5 μL of the resuspended 60% (v/v) acetone pellet. Lanes 1 to 11 contain samples with similar protein quantities from the eluates of the slices of the preparative high pH gel. Lanes marked 'm' contain molecular mass markers (kD). Proteins were stained with Coomassie brilliant blue R-250.

PREPARATIVE GEL ELECTROPHORESIS OF THE PUTATIVE *AVR4* PRODUCT

Proteins present in the 60% (v/v) acetone pellet were subjected to preparative high pH- PAGE and after electrophoresis the proteins were eluted from the gel slices. Figure 1 (lanes 1 to 11) shows the protein composition of the eluates that were obtained from the gel slices. Specific chlorosis- and necrosis-inducing activity was present in the eluates of slices 6 and 7 (Fig. 1, lanes 6 and 7). Slice 6 contained the majority of the putative *avr4* product; necrotic lesions and heavy epinasty were visible at two days after injection into leaflets of near-isogenic line Cf4.

PURIFICATION OF THE PUTATIVE *AVR4* PRODUCT BY HRLC

When the proteins present in the eluate from slice 6, containing the putative *avr4* product, were subjected to HRLC, the absorption profile presented in Figure 2A was obtained. Necrosis-inducing activity eluted in fractions 17 to 21 and was found to be the highest in fraction 17. This fraction caused heavy necrosis and epinasty already one day after injection into leaflets of near-isogenic line Cf4. SDS-PAGE of the proteins present in the collected fractions revealed that fraction 17 contained three proteins, ranging from 5 kD to 14 kD in molecular mass (Fig. 2B, lane 5).

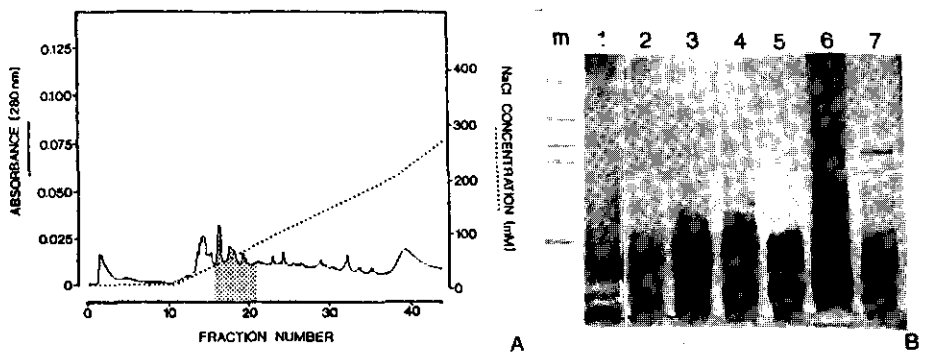

Figure 2. Absorption profile (A) and protein profile (B) obtained by HRLC of the proteins present in the eluate of slice 6 of the preparative high pH, native gel of the 60% (v/v) acetone pellet of apoplastic fluid obtained from the interaction Cf5/race 5. In A the shaded area indicates the fractions that showed necrosis- and chlorosis-inducing activity on near-isogenic line Cf4. Proteins present in 50 μL of the (combined) fractions 2/3 (lane 1), 12/14 (lane 2), 15 (lane 3), 16 (lane 4), 17 (lane 5), 18/19 (lane 6) and 20/21 (lane 7) were separated by SDS-PAGE and stained with silver. The lane marked 'm' contains molecular mass markers (molecular masses are as given in Fig. 1).

INDUCTION OF PATHOGENESIS-RELATED (PR) PROTEINS BY THE PARTIALLY PURIFIED RACE-SPECIFIC ELICITOR

Figure 3 shows that already one day after injection of a sample of the eluate of slice 6 of the preparative high pH gel into leaflets of near-isogenic line Cf4, pathogenesis-related (PR) proteins accumulate in the apoplast (Fig. 3, lane 5). Two and three days after injection necrosis, chlorosis and epinasty of the leaflets was observed and a complete set of PR proteins was induced as well (Fig 3, lanes 6 and 7). Injection of leaflets of near-isogenic line Cf5 with the elicitor preparation did not result in specific induction of PR proteins; the protein compositions of apoplastic fluids isolated from these leaflets were similar to those isolated from leaflets of near-isogenic line Cf4 injected with H_2O (Fig. 3, lanes 1 to 4).

DISCUSSION

Fractionated acetone precipitation, followed by preparative high pH-PAGE and HRLC resulted in a substantial purification of the putative product of avirulence gene *avr4* from apoplastic fluid obtained from the compatible interaction Cf5/race 5. The race-specific elicitor precipitated in 60% (v/v) acetone, migrated on high pH, native gels and bound to an anion exchange column at pH 9.0.

The putative *avr4* product neither migrated as one sharp band on high pH gels, nor did it elute from the MA 7Q column as one protein peak. There is a possibility that the race-specific elicitor, under the non-denaturing conditions that were used to purify it, interacts with other proteins. This will affect the net charge of the elicitor at a certain pH resulting in a 'heterogenous' behaviour. Purification of the putative product of avirulence gene *avr9* was shown to be less elaborate (7, 17). When acetone was added to apoplastic fluid isolated from a compatible interaction involving a race of *C. fulvum* carrying *avr9*, most proteins precipitated, but the *avr9* product remained in the supernatant. The peptide was clearly visible on low pH, native gels as the fastest migrating band, while a relatively pure peptide preparation could be subjected to reverse-phase HPLC. The biological activity of this peptide elicitor, which probably has a tight globular structure, was not affected by the acetonitrile that was used to elute the column.

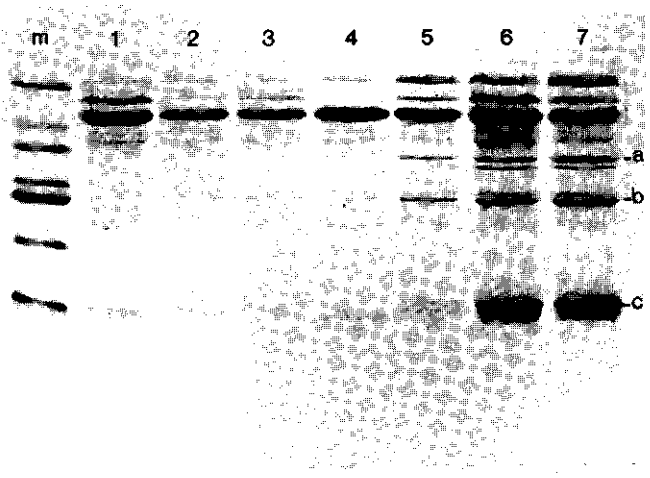

Figure 3. SDS-PAGE of apoplastic fluids isolated from leaflets of near-isogenic lines Cf5 (lanes 2, 3 and 4) and Cf4 (lanes 5, 6 and 7), that were injected with about 100 μL of the dialysed eluate of slice 6 from the preparative high pH gel. Apoplastic fluids were isolated 1 (lanes 2 and 5), 2 (lanes 3 and 6) and 3 days (lanes 4 and 7) after injection of the leaflets. Lane 1 contains apoplastic fluid that was isolated 3 days after injection of leaflets of near-isogenic line Cf4 with H_2O. The 35 kD 1,3-ß-glucanase, 26 kD chitinase and P14 are indicated with a, b and c, respectively. The lane marked 'm' contains molecular mass markers (molecular masses are as given in Fig. 1). Proteins were stained with Coomassie brilliant blue R-250.

Probably the *avr4* product has a higher molecular mass than the *avr9* product and occurs at much lower concentration, making its purification more difficult. Purification to homogeneity might be achieved by hydrophobic interaction chromatography, chromatofocusing or reverse phase chromatography. Microsequencing (separation of the proteins by SDS-PAGE, followed by electroblotting and sequencing of the blotted proteins) (1) might be an alternative

if the putative *avr4* product cannot be purified to homogeneity by standard procedures.

The *avr4* product, produced by race 5 of *C. fulvum* in a compatible interaction, induced HR, epinasty and accumulation of PR proteins in leaflets of near-isogenic line Cf4, which is resistant to race 5 of *C. fulvum*. This observation supports the hypothesis that in the gene-for-gene relationship between tomato and *C. fulvum* host defense is triggered upon molecular recognition of avirulent races of the fungus (8). The peptide encoded by *avr9* directly induces a hypersensitive response in tomato plants carrying resistance gene Cf9. Virulence of races of *C. fulvum* on Cf9 tomato genotypes was shown to result from a deletion of *avr9*; DNA isolated from races of *C. fulvum* that were virulent on Cf9 tomato genotypes and did not produce the elicitor, did not contain sequences homologous to the coding region of *avr9* (22).

From plant pathogenic bacteria that are involved in a gene-for-gene interaction several avirulence genes have been cloned (9). From the plant pathogenic bacterium *Pseudomonas syringae* pv. *glycinea* three avirulence genes (*avrA*, *avrB* and *avrC*) have been cloned and it was shown that these genes are absent in races of the bacterium that are virulent on soybean cultivars carrying corresponding genes for resistance (19, 20). The avirulence genes *avrA*, *avrB* and *avrC* encode proteins (molecular weights 100 kD, 36 kD and 39 kD, respectively), that do not induce HR on the appropriate host cultivars themselves (16, 21), indicating that these proteins have regulatory functions and that other factors are needed for the induction of HR in resistant cultivars of soybean. From *P. s.* pv. *tomato* three avirulence genes have been cloned that elicited HR on appropriate soybean cultivars when they were expressed in *P. s.* pv. *glycinea* (12). One gene was found to correspond to *avrA*, while another, designated *avrD*, was shown to hybridize to DNA isolated from all tested races of *P. s.* pv *glycinea*, although they did not express the phenotype conferred by *avrD*. Research of Kobayashi *et al* (14) revealed that *P. s.* pv. *glycinea* contained a gene that was 93% homologous to *avrD* on DNA level, indicating that this pathovar contains a *P. s.* pv *tomato avrD* gene which has been modified by mutation. The presence of this non-functional allele in *P. s.* pv. *glycinea* enables the bacterium to infect its regular host plant, soybean. The avirulence gene *avrD* encodes a protein of 34 kD in molecular weight (13), which, in combination with

bacterial metabolites, results in a low molecular weight factor that acts as a specific elicitor in soybean (11).

Amino acid sequencing of race-specific elicitors other than the *avr9* product, followed by cloning of their encoding genes, will enable us to determine whether virulence of races of *C. fulvum* is generally caused by a loss of avirulence genes or can also be the result of mutations in functional avirulence genes. Molecular genetic evidence for the validity of the gene-for-gene concept in the tomato-*C. fulvum* interaction can be obtained by transformation of virulent races of *C. fulvum* with functional avirulence genes. Expression of the avirulence genes in the transformants should make them avirulent on tomato cultivars that carry the corresponding resistance genes. Elucidation of the molecular genetics of the tomato-*C. fulvum* interaction could serve as a basis for further research on the molecular mechanisms underlying plant-pathogen specificity.

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CHAPTER 5

IDENTIFICATION OF SEVERAL PATHOGENESIS-RELATED PROTEINS IN TOMATO LEAVES INOCULATED WITH *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*) AS 1,3-ß-GLUCANASES AND CHITINASES

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Identification of Several Pathogenesis-Related Proteins in Tomato Leaves Inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3-β-Glucanases and Chitinases[1]

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ABSTRACT

Inoculation of tomato (*Lycopersicon esculentum*) leaves with *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) results in a marked accumulation of several pathogenesis-related (PR) proteins in the apoplast. Two predominant PR proteins were purified from apoplastic fluid by ion exchange chromatography followed by chromatofocusing. One protein (molecular mass [*M*,] 35 kilodaltons [kD], isoelectric point [pI] ~6.4) showed 1,3-β-glucanase activity, while the other one (*M*, 26 kD, pI ~6.1) showed chitinase activity. Identification of the products that were released upon incubation of the purified enzymes with laminarin or regenerated chitin revealed that both enzymes showed endo-activity. Using antisera raised against these purified enzymes from tomato and against chitinases and 1,3-β-glucanases isolated from other plant species, one additional 1,3-β-glucanase (*M*, 33 kD) and three additional chitinases (*M*, 27, 30, and 32 kD) could be detected in apoplastic fluids or homogenates of tomato leaves inoculated with *C. fulvum*. Upon inoculation with *C. fulvum*, chitinase and 1,3-β-glucanase activity in apoplastic fluids increased more rapidly in incompatible interactions than in compatible ones. The role of these hydrolytic enzymes, potentially capable of degrading hyphal walls of *C. fulvum*, is discussed in relation to active plant defense.

The accumulation of PR[2] proteins upon infection with viroids, viruses, bacteria or fungi has been described for several plant species (28). PR proteins share a number of characteristics such as a relatively low mol wt, accumulation in the apoplast, a high resistance to proteolytic activity, and extreme isoelectric points (28).

The accumulation of PR proteins in tobacco leaves (cv Samsun NN) showing a hypersensitive response to TMV is well documented. The major acidic PR proteins could be resolved on native polyacrylamide gels and were referred to as proteins PR-1a, -1b, -1c, -2, -N, -O, -P, -Q, -R, and -S, in order of decreasing mobility (23). Although much information on the induction and physical properties of these proteins is available, a biological function of some of these proteins has been reported only recently. PR proteins -P and -Q were identified as chitinases and -2, -N, and -O as 1,3-β-glucanases. In addition to these acidic proteins, two basic chitinases and one basic 1,3-β-glucanase were identified (15, 17).

Also, in another species of the Solanaceae, the occurrence of PR proteins has been reported. Kombrink *et al.* (16) identified several PR proteins in potato leaves which were inoculated with *Phytophthora infestans*. Six chitinases and two 1,3-β-glucanases were found in apoplastic fluid as well as in homogenates of infected leaf tissue.

In contrast to *P. infestans* which ramifies through the tissue inter- as well as intracellularly, *Cladosporium fulvum*, the causal agent of tomato leaf mold, is a biotroph that is confined to the apoplast (6). The interchange of proteins and other substances between the two organisms in the apoplast ultimately determines the outcome of the interaction (compatible or incompatible). A marked accumulation of several apoplastic proteins occurs in tomato leaves after inoculation with *C. fulvum*. In addition to the presence of proteinaceous race-specific elicitors (9, 25) and a compatible interaction-specific protein (14), the accumulation of various PR proteins in the apoplast has been reported (10, 11). Thus far the biological function of these tomato PR proteins was unknown.

Here we describe the purification of two predominant PR proteins of tomato one of which is a 1,3-β-glucanase and the other a chitinase. Using antisera raised against 1,3-β-glucanases or chitinases from tomato or other plant species, one additional 1,3-β-glucanase and three additional chitinases could be detected in *C. fulvum* inoculated tomato leaves. Evidence is presented for a differential accumulation of both enzymes in compatible and incompatible *C. fulvum*-tomato interactions.

MATERIALS AND METHODS

Plant, Fungus and Inoculation

The various tomato (*Lycopersicon esculentum*) cultivars carrying different genes for resistance to *Cladosporium fulvum* were grown as described by De Wit and Flach (7).

C. fulvum (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) was subcultured on PDA, and conidial suspensions used to inoculate tomato plants were prepared as described previously (6). Races 4 and 5 of *C. fulvum* were used to inoculate two near-isogenic lines of tomato carrying the resistance genes Cf4

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[2] Abbreviations: PR, pathogenesis related; TMV, tobacco mosaic virus.

(susceptible to race 4 and resistant to race 5) and Cf5 (susceptible to race 5 and resistant to race 4).

Preparation of Apoplastic Fluids and Leaf Homogenates

Entire leaves were infiltrated with distilled water *in vacuo*, and the apoplastic fluids were isolated by centrifugation, according to the method described by De Wit and Spikman (9).

Leaf homogenates were obtained by grinding leaves (after removal of the main veins) in a mortar containing 0.5 M Na-acetate buffer (pH 5.2) (4.0 mL/g fresh weight) and sand. The homogenate was centrifuged for 15 min at 8,000*g*, followed by 30 min at 35,000*g*. The supernatant was collected and analyzed by PAGE.

Determination of Protein

Protein was determined using the method described by Bradford (4), with BSA as a standard.

PAGE

PAGE under low pH, nondenaturing conditions on 10% (w/v) polyacrylamide slab gels was performed following the method described by Reisfeld *et al.* (24). Staining and fixation was as described by Steck *et al.* (27).

SDS-PAGE on 15% (w/v) polyacrylamide slab gels was performed as described by Joosten and De Wit (14). The molecular weight of the various proteins was estimated by co-electrophoresis of marker proteins ranging from 14.2 to 66 kD (Sigma).

Western Blotting and Radioimmunoassays

Western blotting, incubation with antisera, and the radioimmunological detection (with [^{125}I]-labeled protein A) of the antigen-antibody complexes were carried out as described by De Wit *et al.* (10).

1,3-β-Glucanase Assay

To assay for 1,3-β-glucanase activity in apoplastic fluids, proteins in apoplastic fluids were precipitated with 4 volumes of acetone at −20°C. The pellet obtained after centrifugation (5 min, 1,500*g*) was washed twice with 80% (v/v) acetone to remove any reducing sugars. The pellet was suspended in citric acid-phosphate buffer (pH 5.6) and any insoluble material was removed by centrifugation (5 min, 15,000*g*). Appropriate dilutions of the supernatant were used as enzyme solutions. At various steps in the purification procedure protein fractions were assayed for 1,3-β-glucanase activity without pretreatment.

1,3-β-Glucanase activity was assayed by measuring the rate of reducing sugar production with laminarin (Koch-Light) as the substrate. The reaction mixture consisted of 0.4 mL of McIlvaine's citric acid-phosphate buffer (pH 5.6) containing 1 mg/mL laminarin and 0.1 mL of enzyme solution. After 15 min of incubation at 37°C, 0.5 mL of the alkaline copper reagent was added (26) and the mixture was heated at 100°C for 10 min. After cooling on ice, 0.5 mL of the arsenomolybdate reagent (20) was added, followed by 3.0 mL of H_2O after development of the blue color. The absorbance was measured at 660 nm (1). Glucose was used as a standard, and enzyme and substrate alone were included as controls. One katal (kat) was defined as the enzyme activity catalyzing the formation of 1 mol glucose equivalents/s.

Chitinase Assay

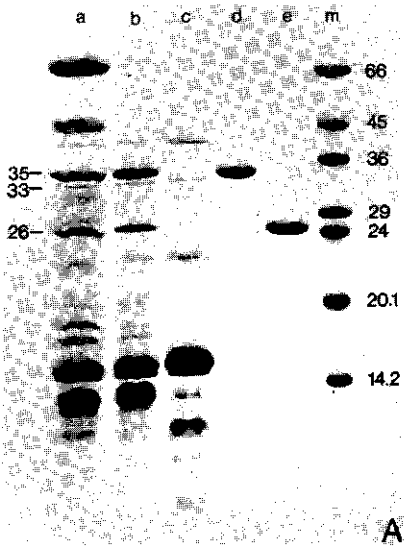
Assays for chitinase activity in apoplastic fluids were carried out without pretreatment of the enzyme solution. Regenerated [^{3}H]chitin, prepared as described by Molano *et al.* (19), was used as a substrate. The reaction mixture (total volume 200 μL) contained 50 mM phosphate buffer (pH 6.4), 0.02% (w/v) NaN_3, 1.7×10^5 cpm of [^{3}H]chitin and apoplastic fluid or purified protein fractions. After incubation at 37°C for 15 min, 0.6 mL of 10% (w/v) TCA was added, and the suspension was centrifuged at 1000*g* for 5 min. The radioactivity of the supernatant (400 μL) was determined by liquid scintillation counting after the addition of 4.0 mL of Aqua Luma Plus (Lumac). Chitinase activity was calculated as described by Boller *et al.* (2) and is expressed as cpm in the released soluble products.

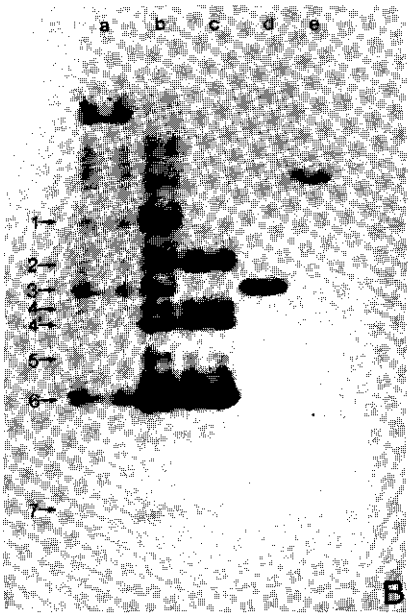
Analysis of 1,3-β-Glucanase and Chitinase Products

The products released by incubation of laminarin or chitin with the purified 1,3-β-glucanase or chitinase were analyzed with a HPLC system equipped with a refractive index detector (Waters). Aminex HPX-87P and HPX-42A columns (300 × 7.5 mm, temperature 85°C) were used for separating monosaccharides and oligosaccharides, respectively.

Purification of a 1,3-β-Glucanase and a Chitinase from Apoplastic Fluids

Four hundred mL of apoplastic fluid, originating from the interaction Cf5/race 5, obtained 14 d after inoculation, was concentrated to about 100 mL by freeze-drying and clarified by centrifugation (10 min, 1000*g*). Proteins were precipitated in 70% (v/v) acetone at −20°C, and the pellet obtained after centrifugation (5 min, 1000*g*) was air dried. The pellet was suspended in 30 mL of 25 mM Na-acetate buffer (pH 4.5) and centrifuged for 5 min at 2000*g*. The clear supernatant was applied to a CM-Sephadex C-25 column (1.6 × 7.5 cm; 15 mL bed volume) equilibrated with 25 mM Na-acetate buffer (pH 4.5), and the column was washed with 40 mL of the same buffer at a flow rate of 15 mL/h to remove the nonbound compounds. The bound proteins were eluted with 0.5 M NaCl in 25 mM Na-acetate buffer, at the same flow rate. The eluate was dialyzed against distilled water, freeze-dried, and the resulting residue was dissolved in 20 mL of 25 mM imidazole-HCl buffer (pH 7.4) and applied to a Polybuffer Exchanger 94 column (Pharmacia, 1.2 × 20 cm, 25 mL bed volume) equilibrated with 25 mM imidazole-HCl buffer (pH 7.4). Elution with 200 mL of Polybuffer 74-HCl (pH 4.0) at 35 mL/h resulted in a p*I* gradient from p*I* 7.0 to p*I* 4.0. The eluent was monitored at 280 nm and the collected fractions (1.8 mL) were analyzed by SDS-PAGE. A Sephadex G-75 column (1.3 × 80 cm, 100 mL bed volume), equilibrated with





H_2O, was used to separate purified proteins from polybuffer. The column was eluted with H_2O at a flow rate of 4 mL/h.

Antiserum Preparation

Fifteen hundred μg of purified 1,3-β-glucanase and 300 μg of purified chitinase were used to raise polyclonal antibodies in rabbits (14). This antiserum was used for immunological detection of proteins on Western blots.

RESULTS

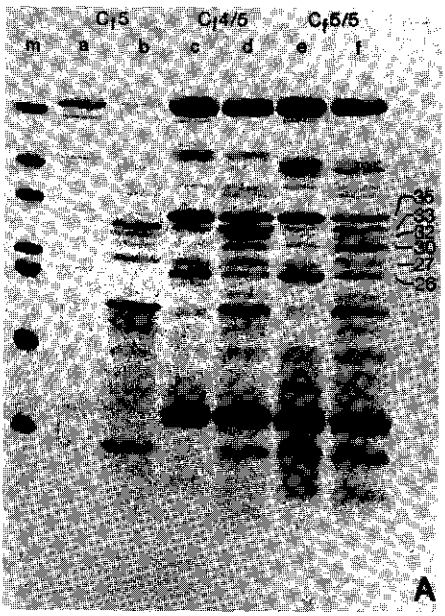
Purification of a 1,3-β-Glucanase and a Chitinase from Apoplastic Fluid

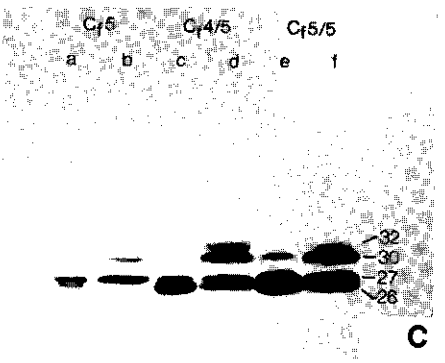
Preliminary immunoblotting experiments with partially purified proteins (M_r 33 kD, and 35 kD, respectively) isolated from apoplastic fluids of *C. fulvum*-infected tomato leaves revealed that these proteins were detected by antiserum raised against PR-O, a 1,3-β-glucanase from tobacco (15). Similarly, another partially purified protein (M_r 26 kD) reacted with antiserum raised against PR-P, a chitinase from tobacco (17). These results prompted us to assay for 1,3-β-glucanase and chitinase activity during the further purification of these proteins.

SDS-PAGE of apoplastic fluid from the interaction Cf5/race 5, obtained 14 d after inoculation resulted in the protein profile presented in Figure 1A, lane a. The 26 kD protein (cross-reacting with the antichitinase serum from tobacco) and the 33 kD and 35 kD proteins (cross-reacting with the anti-1,3-β-glucanase serum from tobacco) are clearly visible on the Coomassie brilliant blue-stained gel. These proteins bound to a CM-Sephadex C-25 column at pH 4.5 and could be eluted with 0.5 M NaCl in 25 mM Na-acetate buffer (pH 4.5) (Fig. 1A, lane b). In the subsequent chromatofocusing step most of the proteins which were eluted from the CM-Sephadex C-25 column did not bind to the column at pH 7.4 (Fig. 1A, lane c). However, the 26 kD and 35 kD proteins bound to the column and were eluted at pH 6.1 and pH 6.4, respectively. The 33 kD protein was not retained by the column. After gel filtration on Sephadex G-75, the 35 kD and 26 kD proteins were purified to apparent homogeneity as far as could be judged from SDS-PAGE (Fig. 1A, lane d and e).

Electrophoresis of the fractions presented in Figure 1A on a low pH, nondenaturing gel resulted in the protein profiles presented in Figure 1B. A number of proteins can be recognized. Protein band 1 represents the compatible interaction-specific protein which has been reported earlier (14), while protein band 7 represents the necrosis-inducing peptide (25).

Figure 1. Protein profiles of fractions obtained at different stages of purification of the 35 kD and 26 kD proteins from apoplastic fluid. PAGE was performed on SDS gel (A) and on low pH, nondenaturing gel (B). Proteins were stained with Coomassie brilliant blue R-250. Shown are profiles of proteins in apoplastic fluid (lane a, 60 μg of protein); proteins bound to Sephadex C-25 and eluted with 0.5 M NaCl (lane b, 35 μg of protein on the SDS gel and 80 μg on the low pH gel); proteins not bound to PBE 94 (lane c, 30 μg) and proteins eluted from PBE 94 within the pH gradient (lane d, 4 μg of the 35 kD protein and lane e, 5 μg of the 26 kD protein). In A, the lane marked 'm' contains M_r markers (M_r given in kD).





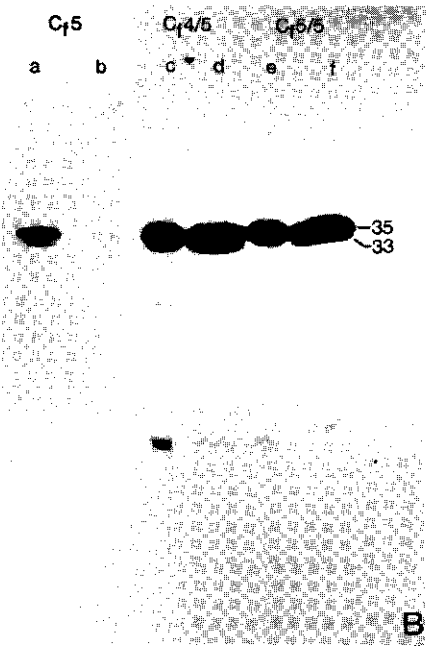


Figure 2. SDS-PAGE (A) of proteins in apoplastic fluids (lanes a, c, and e) and leaf homogenates (lanes b, d, and f) from control plants (Cf5, after 7 d), an incompatible interaction (Cf4/race 5, 7 d after inoculation) and a compatible interaction (Cf5/race 5, 12 d after inoculation). Two replicas of the gel presented in A were blotted onto nitrocellulose, and the blots were incubated with antiserum raised against the purified 1,3-β-glucanase from tomato (B) and antiserum raised against PR-P, a chitinase from tobacco (C). The numbers indicate the M_r in kD of the corresponding protein band. From the uninoculated control plants 300 μL of apoplastic fluid and from the incompatible and compatible interactions 100 μL of apoplastic fluid was applied (100 μL of apoplastic fluid is obtained from 200 mg (fresh weight) of leaf tissue). From the leaf homogenates the proteins extracted from 50 mg (fresh weight) of leaf tissue were electrophoresed. Lanes marked 'm' contain M_r markers (M_r are as given in Fig. 1A).

Proteins 2, 4, 4′, 5, 6, and 7 were not retained by the chromatofocusing column (lane c), in contrast to the 35 kD protein (lane d, representing the earlier reported protein 3) (10) and the 26 kD protein (lane e). The purified proteins were assayed for 1,3-β-glucanase and chitinase activity. The 35 kD protein degraded laminarin (1,3-β-glucanase activity), while the 26 kD protein released soluble products from radioactively labeled regenerated chitin (chitinase activity). Analysis of the products released upon prolonged incubation of laminarin or chitin with the purified 1,3-β-glucanase or chitinase revealed that both enzymes showed endo-activity. Several oligomers were detected, but only low amounts of the

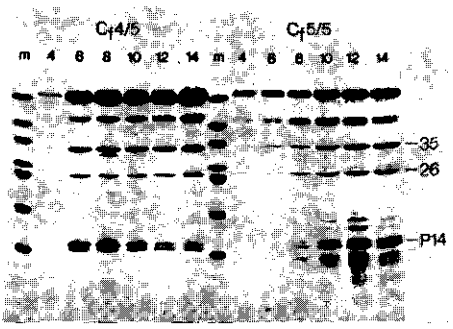


Figure 2. SDS-PAGE of apoplastic fluids obtained 4, 6, 8, 10, 12, and 14 d after inoculation of tomato cultivars Cf4 and Cf5 with race 5 of *C. fulvum*. Each lane contains 200 μL of apoplastic fluid. Lanes marked 'm' contain M_r markers (M_r are as given in Fig. 1A).

monomers were present in the reaction mixtures (results not shown).

Cross-Reaction of Proteins Present in Leaf Homogenates and Apoplastic Fluids with Antisera Raised against 1,3-β-Glucanases and Chitinases

SDS-PAGE of proteins in apoplastic fluids and leaf homogenates obtained from control plants (Cf5), an incompatible interaction (Cf4/race 5), and a compatible interaction (Cf5/race 5) resulted in the protein profiles presented in Figure 2A. The differences in protein patterns between the various samples were mainly quantitative, showing relatively high amounts of the 35 kD 1,3-β-glucanase and 26 kD chitinase in apoplastic fluid obtained from the incompatible and compatible interactions (lanes c and e, respectively). In leaf homogenates of both interactions, the 33 kD and 35 kD proteins are present in more or less equal amounts (lanes d and f, respectively).

Replicates of the gel presented in Figure 2A were blotted onto nitrocellulose and incubated with different antisera. In Figure 2B the blot was incubated with antiserum raised against the purified 1,3-β-glucanase from tomato. The antiserum detected the 35 kD 1,3-β-glucanase and cross-reacted with the 33 kD protein. Incubation of the blot with antiserum raised against a 1,3-β-glucanase (PR-O) from tobacco or antiserum raised against a 1,3-β-glucanase from potato revealed that these antisera had a higher affinity to the 33 kD protein than to the 35 kD protein (results not shown).

In Figure 2C the blot was incubated with antiserum raised against a purified chitinase (PR-P) from tobacco. The antiserum detected the 26 kD chitinase and cross-reacted with a 27, 30, and 32 kD protein. Antiserum raised against the purified 26 kD chitinase from tomato or antiserum raised against a chitinase isolated from bean leaves (2) detected the 26, 30, and 32 kD proteins, but not the 27 kD protein (results not shown).

When samples similar to those presented in Figure 2A were electrophoresed under low pH, nondenaturing conditions,

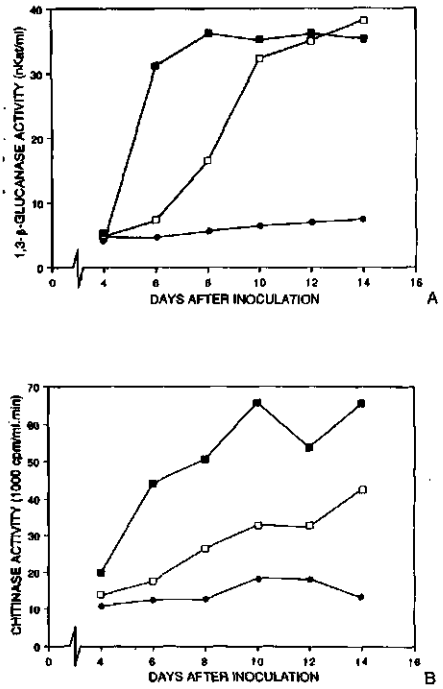


Figure 4. Time course of 1,3-β-glucanase (A) and chitinase (B) activities in apoplastic fluids of leaves of tomato cultivars Cf4 (■) and Cf5 (□) after inoculation with race 5 of *C. fulvum* and of uninoculated Cf5 plants (●). Apoplastic fluids were obtained 4, 6, 8, 10, 12, and 14 d after inoculation, and enzyme activities were determined as described in "Materials and Methods."

antisera raised against the purified 1,3-β-glucanase from tomato and PR-O from tobacco cross-reacted with bands 3 and 5 (Fig. 1B) on a western blot (results not shown). Antisera raised against the purified chitinase from tomato and PR-P from tobacco both detected the chitinase on a western blot and showed low affinity to two proteins near protein band 2 (results not shown).

Differential Accumulation of the 1,3-β-Glucanase and Chitinase in Apoplastic Fluids of Compatible and Incompatible Interactions of *C. fulvum* and Tomato

Apoplastic fluids from tomato cultivars Cf4 and Cf5, inoculated with a conidial suspension of race 5 of *C. fulvum* were isolated at 4, 6, 8, 10, 12, and 14 d after inoculation. SDS-PAGE of the apoplastic fluids revealed that, besides P14 (11), several proteins accumulated about 4 d earlier in the incompatible interaction (Fig. 3). In this interaction (Cf4/race 5) maximal accumulation of the 35 kD 1,3-β-glucanase and

26 kD chitinase had already occurred at 6 d after inoculation and remained more or less constant up to 14 d. The accumulation of P14, however, showed a maximum at 8 d after inoculation and declined afterward. In the compatible interaction (Cf5/race 5) there was a steady increase of the 1,3-β-glucanase and chitinase and several other proteins, up to 14 d after inoculation.

The time course of total 1,3-β-glucanase activity in apoplastic fluid followed the observed accumulation of the 35 kD 1,3-β-glucanase (Fig. 4A). In the incompatible interaction, a fast increase in 1,3-β-glucanase activity occurred, reaching a maximum within 6 d, while in the compatible interaction, after a slow initial increase, the maximal level was reached 4 d later.

The time course of total chitinase activity in apoplastic fluids also revealed a fast increase in the incompatible interaction. The increase in chitinase activity in apoplastic fluids from the compatible interaction was lower than was anticipated from the observed accumulation of the 26 kD chitinase at later stages of infection (Fig. 4B).

Both 1,3-β-glucanase and chitinase activities showed some increase in uninoculated Cf5 plants which were incubated under the same conditions as the inoculated plants. Measurements of time dependent 1,3-β-glucanase and chitinase accumulation in the reciprocal interactions (Cf5/race 4 and Cf4/race 4) gave similar results (results not shown).

DISCUSSION

We have shown that two of the predominant PR proteins which accumulate in the intercellular spaces of tomato leaves after inoculation with *C. fulvum* possess hydrolytic activity. A 35 kD protein showed 1,3-β-glucanase activity, while a protein of 26 kD, possessed chitinase activity.

Young and Pegg (29) isolated three different acidic 1,3-β-glucanases from healthy tomato leaf and stem, but none of them was purified to homogeneity. They also isolated a constitutive chitinase from healthy tomato stem tissue with a M_r of 27 to 31 kD and a pI of 8.5 (22). This enzyme could be similar or related to the 27 kD or 30 kD chitinase in apoplastic fluids or leaf homogenates, which were detected on nitrocellulose blots, but it is different from the 26 kD chitinase because of its different pI and M_r.

Legrand and co-workers (17) showed that four chitinases, isolated from TMV-infected tobacco leaves, had different physicochemical properties but were serologically closely related. Close serological relationships were also found for the four different 1,3-β-glucanases isolated from TMV-infected tobacco leaves (15). All six chitinases that were purified from potato leaves infected by *P. infestans* showed strong cross-reactivity with antiserum raised against a chitinase isolated from bean leaves (16). The two different 1,3-β-glucanases that were purified were serologically closely related as well. These results suggest that 1,3-β-glucanases and chitinases within the Solanaceae, and even between different plant families, are serologically closely related. Assuming that this serological relationship also holds for the chitinases and 1,3-β-glucanases which are induced in tomato by *C. fulvum*, then at least four different chitinases and two different 1,3-β-glucanases are present in inoculated leaves. The chitinases that were detected had a M_r of 26, 27, 30, and 32 kD, while the two 1,3-β-glucanases had M_r of 33 and 35 kD.

Chitinase and 1,3-β-glucanase activities increase in tobacco and potato following infection (15–17). In cucumber, chitinase is induced in response to viral, bacterial and fungal infections (18). In tomato, *Verticillium* or *Fusarium* wilt induced an increase in 1,3-β-glucanase and chitinase activity, which was always higher in the infected susceptible plants, where far more fungal biomass was present than in resistant ones (13, 22). In *Verticillium*-infected tomato plants, the increase in 1,3-β-glucanase and chitinase activity coincided with a levelling off and subsequent reduction of the amount of viable pathogen in the tissue (21). The host glycosidases that were induced by the fungus were found to partially degrade *Verticillium albo-atrum* mycelial wall preparations and could therefore provide a host defense mechanism (30). In tomato, *C. fulvum*, however, induced an increase in 1,3-β-glucanase and chitinase activity in apoplastic fluids that was much faster in the incompatible than in the compatible interaction. In the compatible interaction, it took at least 10 d to reach a level of activity comparable to the level reached at 6 d after inoculation in the incompatible interaction. In the compatible interaction, at 6 d after inoculation, fungal growth is already far more advanced than in the incompatible interaction (11), indicating that in the incompatible interaction the accumulation of the enzymes discussed above is specifically induced by an avirulent race of *C. fulvum* and is not a result of nonspecific stress caused by colonization of the intercellular spaces. In the latter case, one would expect more 1,3-β-glucanase and chitinase in the compatible interaction at this stage of infection. The fast induction of these enzymes, and other PR proteins, in the incompatible interaction, could therefore be the result of recognition by the host of a race-specific elicitor produced by the fungus.

The rapid accumulation of 1,3-β-glucanases and chitinases at the site of penetration in incompatible interactions could play an important role as a defense mechanism of the plant. Many of these hydrolytic enzymes accumulate in the apoplast, as do most of the other PR proteins. These enzymes could protect plants against extracellular fungi like *C. fulvum* but possibly also against pathogenic bacteria most of which grow in the intercellular space.

In hyphal tips of many fungi, 1,3-β-glucans and chitin are exposed at the surface (3) and could be attacked directly by 1,3-β-glucanases and chitinases. The oligosaccharides that are released from the fungal cell wall as a result of this hydrolytic activity could function as elicitors of various plant defense responses (5, 8). Fungal growth could also be inhibited by a disturbance of the balance between synthesis and hydrolysis of hyphal wall material, caused by the extracellular presence of the hydrolytic enzymes (12). In order to test these hypotheses, in future experiments, 1,3-β-glucanases and/or chitinases will be tested for their ability to hydrolyze mycelium and isolated cell walls of *C. fulvum*. It will also be tested whether oligosaccharides released from this substrate are able to induce plant defense responses.

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CHAPTER 6

SUBCELLULAR LOCALIZATION OF A COMPATIBLE INTERACTION-SPECIFIC PROTEIN, CHITINASE AND 1,3-ß-GLUCANASE IN TOMATO LEAF TISSUE INFECTED BY *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA*)

Matthieu H.A.J. Joosten, Jaap C. Bij De Vaate, Birgit P. Loos and Pierre J.G.M. De Wit

ABSTRACT

Antisera raised against the 26 kD chitinase and the 35 kD 1,3-ß-glucanase from tomato (*Lycopersicon esculentum*) were used to study the subcellular localization of these enzymes in tomato leaf tissue infected by *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva*) [Cooke] Cif). In ultrathin leaf sections of the compatible Cf5/race 5 interaction no specific labeling was observed after treatment with the antisera. In the incompatible Cf4/race 5 interaction the hydrolytic enzymes were detected in intercellular spaces, cytoplasm and electron-dense material that was sometimes present in the vacuoles of leaf mesophyll cells. At the site of fungal penetration chitinases and 1,3-ß-glucanases were associated with the electron-dense outer layer of the fungal cell wall. The localized accumulation of the hydrolytic enzymes supports their possible role in active plant defense.

Antiserum raised against the compatible interaction-specific protein P1 detected this protein in the electron-dense matrix that was present between the walls of leaf mesophyll cells and fungal hyphae in the compatible interaction. P1 was not detected in the incompatible interaction and non-inoculated plants. The protein might be an important factor in obtaining or maintaining basic compatibility between plant and fungus.

INTRODUCTION

The accumulation of pathogenesis-related (PR) proteins in several plant species as a response to microbial attack has been reported frequently (20). In tomato several PR proteins accumulate upon infection by viroids (3, 12, 21), viruses (3) or fungi (3, 6, 8, 9, 13, 14). As it has been shown that several PR proteins possess 1,3-ß-glucanase or chitinase activity (11, 14) and are potentially able to inhibit fungal growth (18, 19), the subcellular localization of these enzymes became subject of research (1, 2).

In roots of the susceptible tomato cv Bonny Best infected by *Fusarium oxysporum* f. sp. *radicis lycopersici* (FORL), the accumulation of 1,3-ß-glucanases in host wall areas close to fungal cells and in secondary thickenings of invaded xylem vessels was merely the result of successful tissue colonization (1). In the incompatible tomato cv Larma/FORL interaction, besides labeling of the invaded tissues, also labeling of the uninvaded inner tissues was observed, suggesting that the accumulation of 1,3-ß-glucanases might play a role in effective resistance to FORL. With antibodies raised against the 26 kD tomato chitinase (14), accumulation of chitinase in roots of the FORL-infected tomato cv Bonny Best was mainly detected in fungal wall areas that showed apparent alterations and in host wall areas in the immediate vicinity of fungal cells (2). In the incompatible interaction accumulation of the enzyme occurred earlier and substantial amounts of the chitinase were detected in fungal walls that showed signs of disintegration. It was suggested that a co-ordinated action of 1,3-ß-glucanases and chitinases is needed for effective antifungal activity.

The results of the localization of 1,3-ß-glucanases and chitinases in FORL-infected tomato roots prompted us to determine the *in situ* accumulation of these hydrolytic enzymes in tomato leaves inoculated with virulent or avirulent races of *C. fulvum*. Here we demonstrate that in a compatible tomato-*C. fulvum* interaction no localized accumulation of chitinase and 1,3-ß-glucanase occurs during the early stages of infection. In an incompatible interaction, however, localized accumulation of these hydrolytic enzymes does take place; in certain areas of the leaf tissue the enzymes were detected in intercellular spaces, cytoplasm and electron-dense material present in the vacuoles of mesophyll cells.

Furthermore, the enzymes were found in association with the electron-dense outer layer of hyphal walls that showed substantial degradation.

From apoplastic fluid isolated from a Cf5/race 5 interaction a protein (designated P1) that is specific for compatible interactions between tomato and *C. fulvum* was purified and polyclonal antibodies were obtained (13). Immunolocalization experiments revealed that in the compatible interaction the protein was present in the electron-dense matrix between the walls of leaf mesophyll cells and fungal hyphae. In the incompatible interaction and non-inoculated plants P1 was not detected.

MATERIALS AND METHODS

INOCULATION

Race 5 of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) was subcultured on PDA and a suspension of approximately 5 x 10^6 conidia/mL was used to inoculate near-isogenic lines of tomato (*Lycopersicon esculentum*) Cf4 (resistant to race 5) and Cf5 (susceptible to race 5), as described before (4, 5). Non-inoculated Cf5 plants were kept under the same conditions as the inoculated ones and served as controls.

TISSUE PROCESSING FOR ELECTRON MICROSCOPY

For the immunogold localization experiments tissue samples were taken from leaflets that were just fully expanded. The leaflets were taken from plants that were 8 to 9 weeks old, at 7 days after inoculation. At this time, in the incompatible Cf4/race 5 interaction, fungal development is restricted to a few mesophyll cells (4, 16), while a substantial increase of chitinase and 1,3-ß-glucanase activity has taken place (14). In the compatible Cf5/race 5 interaction fungal growth is far more advanced at 7 days after inoculation, while chitinase and 1,3-ß-glucanase activities are much lower than in the incompatible interaction (14). Leaf tissue samples (1 mm^2) were fixed by immersion in a solution of 1% glutaraldehyde and 0.7% paraformaldehyde in 0.1 M piperazine-1,4-diethanesulfonic acid (PIPES) buffer, pH 7.3, containing 3 mM $CaCl_2$, for 2 h

at room temperature or overnight at 4°C. After rinsing with H_2O the tissue was dehydrated in a graded ethanol series and embedded in Lowicryl K4M at -20°C. The resin was polymerized under UV light of 360 nm. Ultrathin, gold-silver (70 nm) sections were collected on Formvar-coated, 100 mesh, golden grids.

IMMUNOGOLD LABELING

Ultrathin sections of tomato leaves were floated on a drop of 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min and subsequently transferred to antiserum raised against the compatible interaction-specific protein P1 (13), the 26 kD chitinase (14) or the 35 kD 1,3-ß-glucanase (14) from tomato, diluted 1:1000 in PBS-BSA. After incubation for 2 h at room temperature the grids were rinsed with PBS-BSA and subsequently incubated for 1.5 h in protein A-gold (7 or 15 nm in diameter), diluted in PBS-BSA to OD_{520}=0.1, followed by rinsing with PBS-BSA. After fixation in 1% (v/v) glutaraldehyde in PBS for 10 min, the grids were extensively washed with H_2O and contrasted with 4% (w/v) uranyl acetate in H_2O. A silver enhancement step (3 min at room temperature, IntenSE BL, Janssen Biotech) was included before staining with uranyl acetate when colloidal gold of 7 nm in diameter was used. The gold labeled sections were examined with a Zeiss EM 109 electron microscope.

RESULTS

Fixing of the leaf tissue with a combination of glutaraldehyde and paraformaldehyde, followed by embedding in Lowicryl K4M, resulted in a moderate ultrastructural preservation, however, the immobilisation of the antigens and maintenance of their antigenicity was satisfactory.

IMMUNOGOLD LOCALIZATION OF P1, CHITINASE AND 1,3-ß-GLUCANASE IN NON-INOCULATED AND INOCULATED TOMATO LEAF TISSUE

Non-inoculated Cf5 plants

Observation of sections of leaf tissue of non-inoculated tomato plants that were kept under the same conditions as the inoculated ones, revealed that the mesophyll cells generally contained one large vacuole and a thin layer of cytoplasm that was closely appressed to the cell wall. Incubation of tissue sections with antiserum raised against P1, chitinase or 1,3-ß-glucanase resulted in a very low background labeling of intercellular spaces, cell walls, cytoplasm and vacuoles (results not shown).

Compatible interaction Cf5/race 5

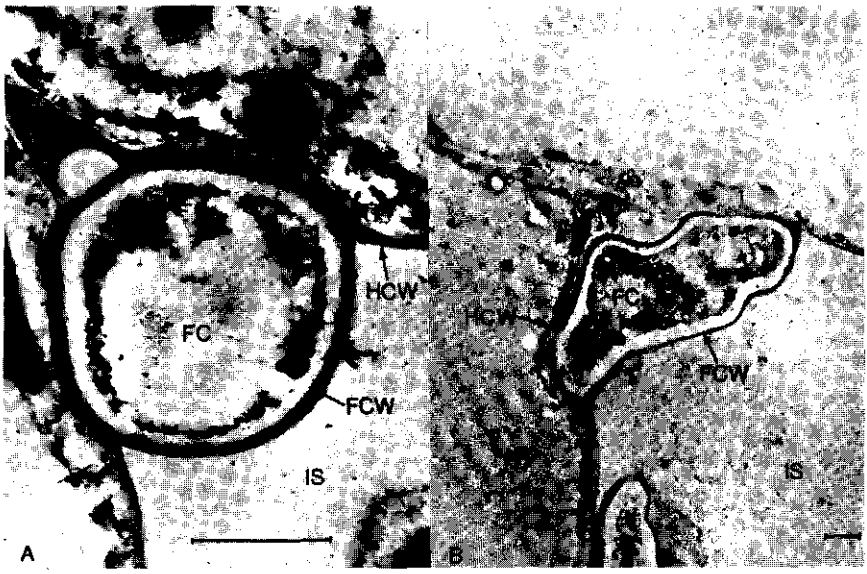

Figure 1. Transmission electron micrographs of leaf tissue of near-isogenic line Cf5 infected by race 5 of *C. fulvum* (compatible interaction). Fungal hyphae, circular (A) or irregular (B) in shape, are growing in close contact with the plant cell wall. Magnification x 12,000 (A) and x 3,000 (B); bar = 1 μm. FC, fungal cell; FCW, fungal cell wall; HCW, host cell wall; IS, intercellular space.

Examination of ultrathin sections of leaf tissue obtained from the compatible interaction Cf5/race 5 revealed that 7 days after inoculation substantial colonization of the intercellular spaces had occurred. Host wall areas in close contact with fungal hyphae usually showed indentation. The hyphae, filled with electron-dense material, were circular (Fig. 1A) or irregular (Fig. 1B) in shape. The outer layer of the hyphal wall consisted of electron-dense material, while the thick inner layer was more electron-translucent (see also 17).

Sections of infected leaf tissue, obtained 7 days after inoculation, treated with antiserum raised against the compatible interaction-specific protein P1, showed heavy deposition of gold particles in the electron-dense matrix between host cell walls and fungal hyphae (Fig. 2).

Figure 2. Transmission electron micrograph of leaf tissue of near-isogenic line Cf5 infected by race 5 of *C. fulvum* (compatible interaction). Labeling was performed with antibodies raised against the compatible interaction-specific protein P1 and protein A-gold (15 nm). Gold particles predominantly accumulate over the electron-dense matrix between the fungal and host cell wall. Magnification x 20,000; bar = 0.5 μm. For abbreviations see legend of Figure 1.

Only minor labeling was detected when sections were treated with antiserum raised against chitinase (Fig. 3) or 1,3-ß-glucanase (result not shown). Except for some labeling on the outside of the hyphal wall no significant labeling was detected elsewhere.

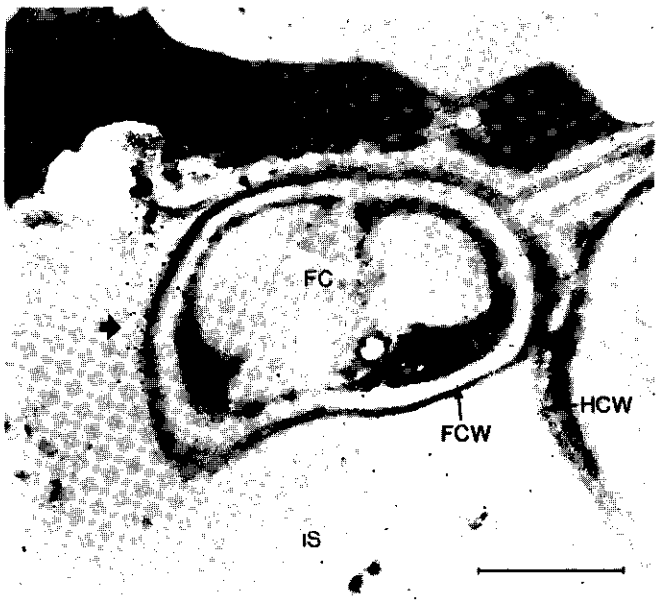

Figure 3. Transmission electron micrograph of leaf tissue of near-isogenic line Cf5 infected by race 5 of *C. fulvum* (compatible interaction). Labeling was performed with antibodies raised against the 26 kD tomato chitinase and protein A-gold (15 nm). Besides some labeling near the electron-dense outer layer of the hyphal wall (arrow), no specific labeling occurred. Magnification x 12,000; bar = 1 μm. For abbreviations see legend of Figure 1.

Incompatible interaction Cf4/race 5

In the incompatible interaction Cf4/race 5 the intercellular spaces were not colonized; growth of the hyphae stopped about two days after penetration of the stomata and fungal development remained restricted to a few mesophyll cells (4, 16, 17). Generally the amount of fungal material present in the leaves that were used for the localization experiments was low and it was very difficult to find fungal hyphae.

When ultrathin sections of leaf tissue, obtained 7 days after inoculation, were incubated with antiserum raised against P1, no specific labeling was observed (results not shown). Specific labeling was found when sections of inoculated leaf tissue were incubated with antiserum raised against chitinase or 1,3-ß-glucanase. In some parts of the host tissue specific labeling of intercellular spaces (Fig. 4A) and cytoplasm (Fig. 4B) was observed. Occasionally specifically labeled electron-dense material that was present in the vacuoles of the mesophyll cells was observed (Fig. 4B). Probably these structures correspond to the "polymorphic inclusions" and "inclusion-bodies" detected by Benhamou *et al* (1) and Vera *et al* (22, 23), respectively.

When fungal hyphae were detected they were devoid of cytoplasm, and the cell wall appeared to be partially degraded. Incubation of ultrathin sections of leaf tissue that contained fungal material with rabbit pre-immune serum, followed by protein A-gold, did not reveal any specific labeling (Fig. 5A). Only the electron-dense outer layer of the hyphal wall could be recognized. Incubation of similar sections with antiserum raised against chitinase resulted in heavy deposition of gold particles on this electron-dense layer (Fig. 5B). Figure 5C shows another example of a labeled hypha that was observed in the incompatible interaction. The heavily indented plant cell wall showed no labeling, while the outer layer of the wall of the fungal cell, which had lost its cytoplasm, showed substantial labeling. Treatment of similar sections with antiserum raised against 1,3-ß-glucanase gave identical results; the electron-dense outer layer of the fungal cell wall showed substantial labeling (results not shown).

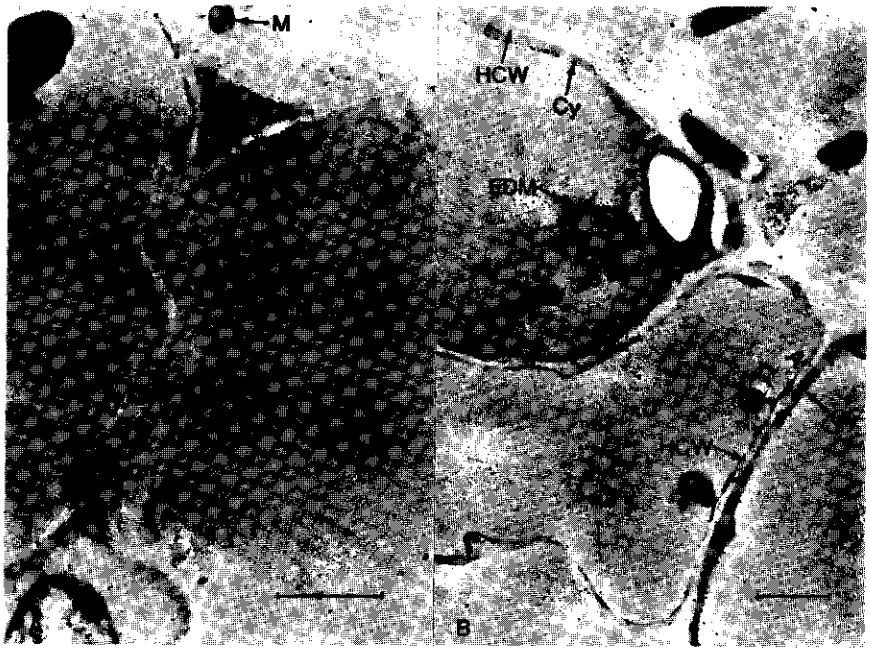

Figure 4. Transmission electron micrographs of leaf tissue of near-isogenic line Cf4 infected by race 5 of *C. fulvum* (incompatible interaction). Labeling was performed with antibodies raised against the 26 kD tomato chitinase and protein A-gold (7 nm). Specific labeling of intercellular spaces (A), cytoplasm (B) and electron-dense material (B) was observed. Magnification x 4,400 (A) and x 3,000 (B); bar = 2 μm. Cy, cytoplasm; EDM, electron-dense material; M, mitochondrion; other abbreviations as in Figure 1.

DISCUSSION

In compatible interactions between tomato and *C. fulvum* initially host cells do not respond to the presence of the pathogen. Fungal hyphae are closely appressed to the host cell walls and the indentation of these walls causes an increase of the area of contact between plant and fungus, which could be important for an

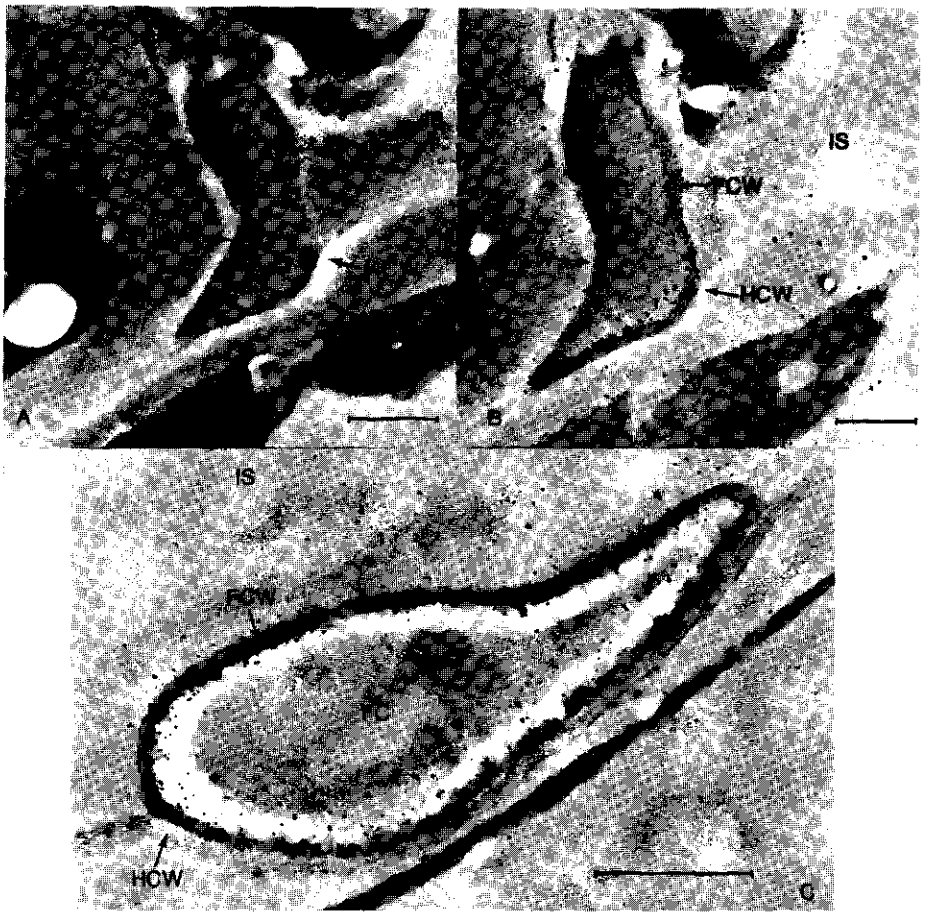

Figure 5. Transmission electron micrographs of leaf tissue of near-isogenic line Cf4 infected by race 5 of *C. fulvum* (incompatible interaction). Labeling was performed with pre-immune serum and protein A-gold (7 nm, A) and with antibodies raised against the 26 kD tomato chitinase and protein A-gold (7 nm (B) and 15 nm (C)). No labeling occurred after treatment with pre-immune serum (A). Specific labeling of the electron-dense layer of the hyphal cell wall occurred when ultrathin sections were incubated with serum raised against the 26 kD chitinase (B, C). Magnification x 7,000 (A, B) and x 12,000 (C); bar = 1 μm. For abbreviations see legend of Figure 1.

efficient uptake of nutrients from the host. No haustorium-like structures have been identified (4, 16, 17). The fungus presumably obtains carbohydrates (15), amino acids and other nutrients from the apoplast of the tomato leaf. Our observation that the compatible interaction-specific protein P1 (13) is predominantly present in the electron-dense matrix between host and fungal cell wall supports the hypothesis that this protein is an important factor of basic compatibility. Possibly this protein is involved somewhere in the process of establishing and/or maintaining contact between host and fungus and absorbing of nutrients from the host cells.

In incompatible interactions between tomato and *C. fulvum* host cells show a rapid response upon recognition of the pathogen, resulting in a restriction of fungal development to a few mesophyll cells. The response of the plant consists of host cell necrosis, deposition of polyphenols and callose, leakage of electrolytes, accumulation of phytoalexins, and the production of PR proteins and hydrolytic enzymes such as 1,3-ß-glucanases and chitinases (5, 7, 8, 9, 10, 14, 16, 17). The fast increase of apoplastic 1,3-ß-glucanase and chitinase activities in the incompatible interaction supports the hypothesis that these plant hydrolytic enzymes are an important component of the plant defense mechanism (14), but no evidence was obtained about the accumulation of these enzymes at the site of fungal penetration. In this report we have shown that in the incompatible Cf4/race 5 interaction indeed chitinases and 1,3-ß-glucanases accumulate around the site of penetration and bind to the fungal wall. To some extent degradation of the hyphal wall and loss of cytoplasm was observed. In the compatible Cf5/race 5 interaction no specific labeling was observed when leaf sections where incubated with antibodies raised against tomato chitinase or 1,3-ß-glucanase. These results are in agreement with observations of Benhamou *et al* (1, 2), who showed that in roots of a tomato cultivar resistant to FORL, accumulation of host chitinases and 1,3-ß-glucanases appeared to be an early event associated with the reduction of fungal growth while in a compatible interaction the accumulation was a result of general stress caused by successful tissue colonization. The localized accumulation of host hydrolytic enzymes in the incompatible interaction does not prove that these enzymes restrict fungal colonization, but it clearly provides additional support for their role in active plant defense. The relative importance of the hydrolytic enzymes in the total defense arsenal of the plant remains to be determined.

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CHAPTER 7

PURIFICATION AND SEROLOGICAL CHARACTERIZATION OF THREE BASIC 15-KILODALTON PATHOGENESIS-RELATED PROTEINS FROM TOMATO

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Purification and Serological Characterization of Three Basic 15-Kilodalton Pathogenesis-Related Proteins from Tomato[1]

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ABSTRACT

In tomato (*Lycopersicon esculentum*) several acidic and basic apoplastic pathogenesis-related (PR) proteins are induced upon inoculation with virulent or avirulent races of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif). One of the most predominant and best characterized tomato PR proteins is P14, a basic protein that shows homology to the tobacco (*Nicotiana tabacum*) PR-1 protein family. To investigate whether, by analogy with these tobacco PR-1 proteins, P14 also belongs to a family of differently charged isomers, the abundantly occurring PR proteins with molecular masses around 15 kilodaltons (kD) were purified from apoplastic fluids isolated from *C. fulvum*-infected tomato. Three basic proteins migrating similarly to P14 on sodium dodecyl sulfate polyacrylamide gels were purified to homogeneity by gel filtration followed by high resolution liquid chromatography. Two proteins (15.5 kD, isoelectric point [pI] 10.9 and 10.7 appeared to be serologically related to each other and to the tobacco PR-1 proteins. A third protein (15 kD, pI 10.4) was not serologically related to any other tomato PR protein but was found to be related to PR-R from tobacco.

In tomato the synthesis of several types of acidic and basic PR[2] proteins is induced upon infection by viroids (1, 12), viruses (1), or fungi (1, 3, 7, 9, 10, 16, 17, 23). Besides these biotic inducers, various chemical treatments also have been shown to induce the accumulation of PR proteins (3, 12).

One of the most abundant PR proteins in induced tomato plants is P14. This protein, named after its apparent molecular mass of 14 kD, was first identified in extracts of PSTV-infected tomato leaves (1, 2). From those extracts P14 has been purified (2) and its primary structure was determined (21). While an enzymatic function has been ascribed to some of the tomato PR proteins (11, 17, 28), the function of P14 remains to be elucidated. However, immunogold localization of P14 in citrus exocortis viroid-infected tomato leaves revealed that the occurrence of the protein was associated mainly with electron-dense material in the intercellular spaces and with vacuolar 'inclusion bodies' (29, 30). Hence, it was concluded that P14 could be involved in cell degeneration during senescence or pathogenic attack.

In leaves of TMV-infected 'Samsun NN' and 'Xanthi-nc' tobacco, three serologically related, extracellular, acidic PR proteins (PR-1a, -1b, and -1c) with similar molecular mass (15 kD) but different pI values have been identified (15, 24, 27). The primary structures of the three PR-1 proteins show a homology of approximately 90% (4). Antibodies to P14 cross-react with at least one PR-1 protein (23), and the amino acid sequence of P14 shows a 60% homology with the tobacco PR-1b protein (4).

Previously we have shown that, after inoculation of tomato leaves with *Cladosporium fulvum*, P14 accumulates significantly faster in incompatible than in compatible interactions and that its accumulation can be used as an early marker of incompatibility (9). Antibodies raised against P14 purified from PSTV-infected tomato leaves (2) were shown to detect three different protein bands on western blots of low pH, nondenaturing polyacrylamide gels containing apoplastic proteins from *C. fulvum*-infected tomato (10). To investigate whether, by analogy with the tobacco PR-1 proteins, P14 consists of a family of differently charged isomers with similar molecular masses, the most predominant 15-kD tomato PR proteins were studied in more detail. In this report we describe the purification and characterization of three basic proteins (P2, P4, and P6 according to their identification on low pH, native gels) with molecular masses of about 15 kD from apoplastic fluid isolated from *C. fulvum*-infected tomato leaves. Proteins P4 and P6 appeared to be serologically related to each other and to the tobacco PR-1 proteins. Protein P2, however, was not serologically related to any other protein present in apolastic fluid isolated from *C. fulvum*-infected tomato, but showed serological relationship to tobacco PR-R.

MATERIALS AND METHODS

Inoculation and Isolation of Apoplastic Fluid

Tomato

Race 5 of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) was subcultured on potato dextrose agar and a

[1] This research was carried out in the framework of contract BAP-0074-NL of the Biotechnology Action Programme of the Commission of the European Communities.

[2] Abbreviations: PR, pathogenesis-related; kD, kilodalton; PSTV, potato spindle tuber viroid; TMV, tobacco mosaic virus; pI, isoelectric point; IEF, isoelectric focusing.

conidial suspension was used to inoculate tomato (*Lycopersicon esculentum* L.) cv Cf4 (resistant to race 5) or cv Cf5 (susceptible to race 5) according to methods described previously (5, 6). Ten days (incompatible interaction) or 14 d (compatible interaction) after inoculation, apoplastic fluids were isolated by infiltration of whole leaflets with distilled water *in vacuo*, followed by centrifugation, according to the method described by De Wit and Spikman (8).

Tobacco

Eight- to nine-week-old tobacco plants (*Nicotiana tabacum* cv Samsun NN) were inoculated with TMV (strain WU1, 3 μg/mL) and 7 d later apoplastic fluid was isolated from inoculated leaves according to the procedure described by De Wit and Spikman (8). Leaves were infiltrated with 50 mM potassium phosphate buffer (pH 7), containing 0.1% 2-mercaptoethanol. After centrifugation the apoplastic fluid was concentrated by ultrafiltration (YM10 filter, Amicon) to a protein content of 300 to 500 μg/mL.

Electrophoresis

PAGE under low pH, nondenaturing conditions to resolve basic proteins was performed according to the method described by Reisfeld *et al.* (25). SDS-PAGE was performed as described by Joosten and De Wit (16). Molecular mass markers ranging from 14.2 to 66 kD (Sigma) were coelectrophoresed to estimate molecular masses of the various proteins. PAGE under high pH, native conditions to resolve acidic proteins was similar to SDS-PAGE, with the exception that SDS was omitted in all buffers. The sample buffer did not contain 2-mercaptoethanol.

Two-dimensional PAGE was performed basically according to the method described by Hogue and Asselin (13) with some minor modifications. Slices from the first-dimension gels (low or high pH, native gels) were kept in distilled water overnight and equilibrated with SDS sample buffer in a test tube at room temperature for 10 min, followed by immersion in boiling water for 3 min. The gel pieces were placed on top of a second gel to perform regular SDS-PAGE.

IEF was carried out on commercial ampholine polyacrylamide gels (LKB) according to the manufacturer's protocol. pI markers, ranging from pI 4.75 to pI 10.6 (LKB), were coelectrophoresed to estimate the pI of the various proteins.

Proteins on polyacrylamide gels were stained with Coomassie brilliant blue R-250 as described by Joosten and De Wit (16), IEF gels were fixed and stained according to the LKB protocol. Silver staining of two-dimensional gels was performed according to the method described by Merril *et al.* (22).

Western Blotting

Western blotting and subsequent incubation of the blots with antisera were performed as described by De Wit *et al.* (10). Detection of the antigen-antibody complexes was carried out with the Bio-Rad Immun-Blot goat anti-rabbit alkaline phosphatase assay kit.

Purification of the 15-kD Tomato PR Proteins

Proteins present in 240 mL of apoplastic fluid, obtained from 500 g leaves from the incompatible interaction Cf4/race 5 at 10 d after inoculation, were precipitated over night in 60% (v/v) acetone at −20°C. The pellet obtained after centrifugation (5 min, 1,000*g*) was air-dried and suspended in 3 mL of 50 mM Tris-HCl buffer (pH 7.5), containing 100 mM NaCl (elution buffer). After centrifugation (15 min, 13,000*g*), the clear supernatant was applied to a Sephadex G-50 column (2.6 × 100 cm) equilibrated with elution buffer and the column was eluted at a flow rate of 10 mL/h. The eluent was monitored at 280 nm and collected fractions (5 mL) were combined on the basis of their UV absorption profile and analyzed by SDS-PAGE. The fraction containing the PR proteins with molecular masses of about 15 kD was dialyzed against distilled water, freeze-dried and dissolved in 20 mM Mes buffer (pH 6.0) (buffer A). Aliquots of 200 μL were subjected to high resolution liquid chromatography (Bio-Rad), on a MA 7S cation exchange column (Bio-Rad) that was equilibrated with buffer A. After loading of the protein sample, the column was washed with buffer A at a flow rate of 1.5 mL/min for 3 min and the bound proteins were subsequently eluted with a linear NaCl gradient (0–200 mM, in 10 min) in buffer A at the same flow rate (fractions of 0.75 mL were collected). After analysis by SDS-PAGE, fractions containing the purified 15 kD proteins were freeze-dried, dissolved in 100 μL of distilled water and dialyzed against distilled water, in a Pierce System 100 Microdialyzer. Several repeated runs yielded amounts of protein that were sufficient for the analysis by PAGE and preparation of antisera in rabbits (300 μg of each of the purified proteins were used to raise polyclonal antibodies [16]).

Purification of PR-R from Tobacco

PR-R was purified essentially according to Kauffmann *et al.* (18, 19). Approximately 400 g of infected leaves were ground in a Waring Blendor in 600 mL of 0.5 M sodium acetate buffer (pH 5.2) containing 15 mMM 2-mercaptoethanol. The extract was centrifuged at 6,000*g* for 15 min and at 15,000*g* for 50 min. The supernatant was loaded onto a Sephadex G-25 column (11 × 60 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.2). The protein fraction was allowed to stand at 4°C overnight and centrifuged at 20,000*g* for 50 min. The supernatant was loaded onto a S-Sepharose fast flow column (5 × 5 cm; Pharmacia) equilibrated with 20 mM sodium acetate buffer (pH 5.2). The nonbound protein fraction was dialyzed against a 20 mM Tris-HCl buffer (pH 8.0) and loaded onto a Q-Sepharose fast flow column (5 × 5 cm; Pharmacia) equilibrated with the same pH 8.0 buffer. The nonbound protein fraction was concentrated by ultrafiltration (YM10 filter, Amicon) to 300–500 μg protein/mL, dialyzed against 25 mM diethanolamine-HCl buffer (pH 8.3) and loaded onto a prepacked fast protein liquid chromatography column (mono P, HR 5/20; Pharmacia) equilibrated with the same pH 8.3 buffer. Bound proteins were eluted with a pH gradient generated by a buffer containing 7% Polybuffer 74 and 3% Polybuffer 96 (both from Pharmacia), and adjusted

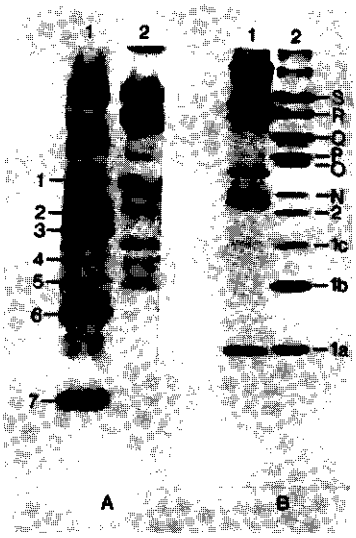

Figure 1. PAGE on low pH (A) and high pH (B), native gels of apoplastic fluids isolated from a compatible Cf5/race 5 interaction (lanes 1) or TMV-infected Samsun NN tobacco (lanes 2). From tomato, 200 μL of apoplastic fluid was electrophoresed, while from tobacco 100 μL (low pH) and 50 μL (high pH) were electrophoresed. Proteins were stained with Coomassie brilliant blue.

to pH 5.0 with HCl. The peak fractions were collected and analyzed by PAGE.

RESULTS

To characterize P14 and the P14-like proteins of tomato in greater detail and to establish possible relationships among these PR proteins and those of tobacco, proteins in the apoplastic fluid of the compatible tomato Cf5/race 5 interaction and of TMV-infected Samsun NN tobacco were analyzed by PAGE. Figure 1 A shows the separation of proteins present in the apoplastic fluid from *C. fulvum*-infected tomato (lane 1) and from TMV-infected tobacco (lane 2) on a nondenaturing, low pH gel. The tomato proteins are labeled according to literature (10, 17). Except for P1 and P7, the synthesis of an identical set of PR proteins is induced in the incompatible tomato Cf4/race 5 interaction (Fig. 2A, first dimension). Proteins P1 and P7 represent the compatible interaction-specific protein (16) and the necrosis inducing peptide (26), respectively. P3 and P5 have been identified previously as 1,3-β-glucanases (17). As yet, the basic proteins in the apoplastic fluid of TMV-infected tobacco (Fig. 1A, lane 2) have not been analyzed in detail. Figure 1B shows the protein patterns of the same two samples after electrophoresis on a nondenaturing, high pH gel. The labelling of the tobacco proteins is according to literature (14, 27). Proteins PR-2, -N, and -O with similar molecular masses of 35 to 40 kD have been shown to be 1,3-β-glucanases (18), whereas proteins PR-P and -Q molecular mass 28 kD have been identified as chitinases (14, 20). No enzymatic function could be attributed to the PR-1 proteins or to PR-S and -R. Upon analysis on a denaturing SDS gel the latter protein splits up into two bands, PR-r_1 (molecular mass 14.5 kD) and PR-r_2 (molecular mass 13 kD), respectively. Probably this PR-R corresponds to PR-r_1 and -r_2 reported by Kauffmann *et al.* (19), PR-R′ reported by Pierpoint (24), PR-R reported by Van Loon et al.

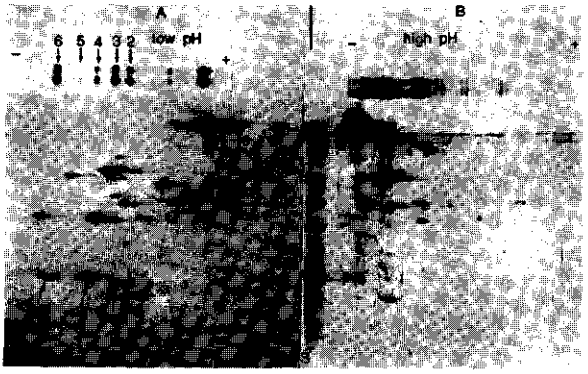

Figure 2. Two-dimensional PAGE of apoplastic fluid isolated from the incompatible interaction Cf4/race 5. Apoplastic fluid (300 μL) was freeze-dried and subjected to native low pH (A) or high pH (B) PAGE (first dimension) and subsequently separated by SDS-PAGE (second dimension). As a control 100 μL of apoplastic fluid was run in the second dimension. In section A the three basic 15 kD proteins are marked P2, P4, and P6. Section B shows that no acidic 15-kD proteins were present in the apoplastic fluid. In the marker lane the numbers indicate the position of the molecular mass markers (kD). The first dimension gel strips were stained with Coomassie brilliant blue R-250 while the second dimension gel was stained with silver.

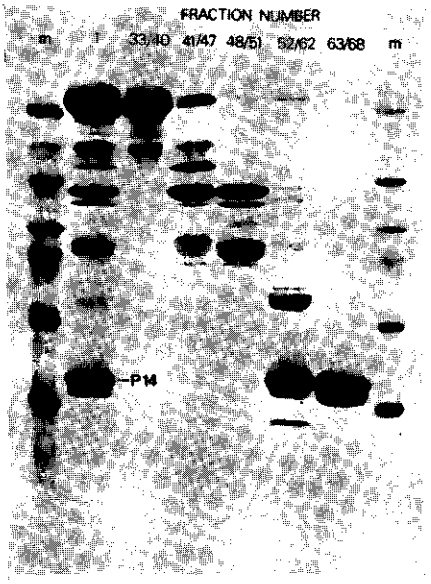

Figure 3. SDS-PAGE of the fractions that were collected with gel filtration on Sephadex G-50 of proteins present in apoplastic fluid from the incompatible interaction Cf4/race 5. Lane 1 contains 150 μL of apoplastic fluid, the numbers indicate the fractions that were combined based on the absorption profile of the eluate (200 μL of the combined fractions was applied to the gel). Fractions 63 to 68 contained the 15-kD PR proteins and were used for further purification. Lanes marked 'm' contain molecular mass markers (molecular masses are as given in Fig. 2). The gel was stained with Coomassie brilliant blue R-250.

(27) and 9b and 9c reported by Hogue and Asselin (13). The PR protein named R by Kauffmann *et al.* (19) probably corresponds to PR-S (molecular mass 25 kD) described by Van Loon *et al.* (27).

Analysis of the proteins in the apoplastic fluid from tomato by two-dimensional electrophoresis revealed the presence of three basic PR proteins (P2, P4, and P6) (10) with mobilities similar to P14 in the second dimension (Fig. 2A). One of the proteins (P2) had a slightly higher mobility, while the two others (P4 and P6) migrated the same distance as P14. No acidic proteins migrating similarly to P14 were detected (Fig. 2B).

Purification of the Basic 15-kD PR Proteins

Gel filtration of the proteins in apoplastic fluid isolated from the incompatible interaction Cf4/race 5 on Sephadex G-50 separated the 15-kD PR proteins from other proteins. Figure 3 shows that the 15-kD proteins were the last to be eluted from the column, in fractions 63 to 68. These fractions were combined and the proteins were further purified by high resolution liquid chromatography. Loading of the proteins present in 1 mL of the combined fractions onto the MA 7S column resulted in an elution profile with three distinct protein peaks. One peak was slightly retarded by the column and eluted before the start of the NaCl gradient, while the other two eluted at the start and the end of the NaCl gradient, respectively. Three repeated runs, representing 5 mL of the combined fractions each, yielded sufficient quantities of the three proteins for further studies.

Characterization of the Purified 15-kD Proteins by PAGE

Analysis of the individual protein peaks by low pH, native PAGE revealed that they corresponded to bands P2, P4, and P6, respectively (Fig. 4A, lanes 3, 4, and 5). The minor bands visible in lanes 3 to 5 are probably caused by artefacts inherent to native, low pH PAGE. SDS-PAGE of the same samples showed that the proteins were purified to homogeneity (Fig. 4B). Proteins P4 and P6 (lanes 4 and 5) migrated to the same position as P14 (15.5 kD), while protein P2 (lane 3) migrated slightly faster (15 kD).

IEF revealed that most of the proteins present in apoplastic fluid originating from the incompatible Cf4/race 5 interaction are acidic (Fig. 4C, lane 1). The purified proteins P2, P4, and P6 appeared to be very basic; their pI values were estimated to be 10.4, 10.9, and 10.7, respectively (lanes 3, 4, and 5).

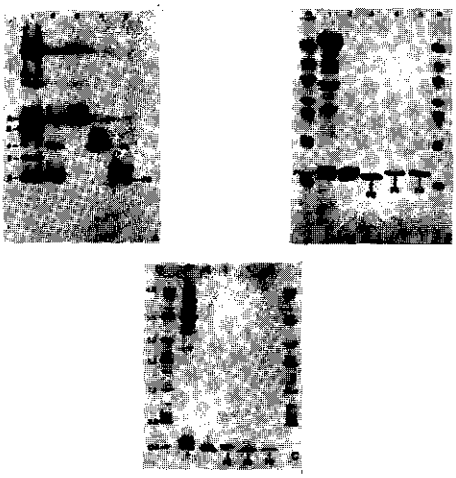

Figure 4. PAGE of the 15-kD PR proteins purified by high resolution liquid chromatography on low pH, native gel (A), SDS gel (B) and IEF gel (C). Besides the original apoplastic fluid (lanes 1, 200 μL) and the combined fractions (63 to 68) of the gel filtration column (lanes 2, 100 μL), samples of 5 μg of the individual protein peaks were applied to the gels. Lanes marked 'm' in Figure 4B contain molecular mass markers (molecular masses are as given in Fig. 2). On the IEF gel (C) pI markers were coelectrophoresed; the pI of the markers is indicated. The gels were stained with Coomassie brilliant blue R-250.

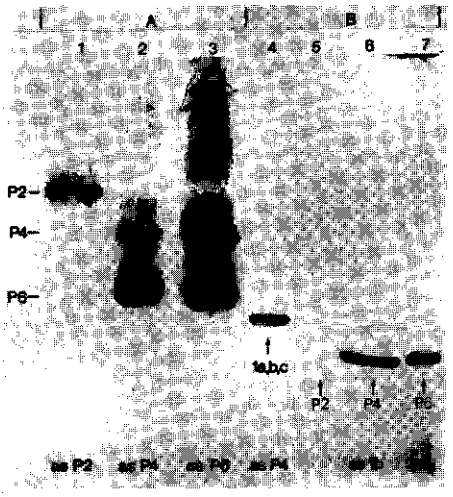

Figure 5. Immunoblots of PR proteins electrophoresed on low pH, native gel (A) or SDS gel (B). Besides the apoplastic fluids from *C. fulvum*-infected tomato (200 μL, lanes 1, 2, and 3) and TMV-infected tobacco (50 μL, lane 4), 5 μg samples of the purified proteins P2, P4, and P6 (lanes 5, 6, and 7, respectively) were electrophoresed. The blots were probed with antiserum (as) raised against tomato P2, P4, P6, or tobacco PR-1b.

Proteins in apoplastic fluid isolated from the compatible interaction Cf5/race 5 subjected to the same purification procedures showed similar results (not shown). In compatible interactions, the same set of PR proteins is induced upon inoculation, but they appear later than in incompatible interactions (9, 10, 17).

Immunological Relationships between PR Proteins P4 and P6 from Tomato and PR-1a, -1b, and -1c from Tobacco

In order to characterize the purified proteins in more detail, antibodies were raised to P2, P4, and P6 and used to probe a Western blot of a native, low pH gel loaded with proteins present in intercellular fluid isolated from *C. fulvum*-infected tomato. Antibodies raised against P2 detected P2, while no cross-reactions with other proteins occurred (Fig. 5, lane 1). Probing the blots with antibodies to P4 and P6 revealed cross-reactions with P6 and P4, respectively, indicating that these proteins are serologically related (Fig. 5, lanes 2 and 3).

Antibodies to P4 also cross-reacted with PR-1a, -1b, and -1c from tobacco (Fig. 5, lane 4) and antibodies raised against PR-1b cross-reacted with purified P4 and P6, but not with purified P2 (Fig. 5, lanes 5, 6, and 7). Similar results were obtained when antibodies raised against P6, PR-1a or -1c were used (data not shown).

Immunological Relationship between PR Protein P2 from Tomato and PR-R from Tobacco

Probing a blot of a nondenaturing, high pH gel loaded with proteins from the apoplastic fluid of TMV-infected tobacco with antiserum raised against P2 revealed a specific reaction with protein PR-R (Fig. 6, lane 3). This finding prompted us to purify this tobacco protein. An extract of TMV-infected tobacco was desalted and passed over an S- and Q-Sepharose column, respectively. Proteins that did not bind to either column were fractionated by chromatofocusing on a mono-P column. Analysis of the individual peak fractions by nondenaturing, high pH PAGE revealed that one of them contained virtually pure PR-S and another one PR-R. Under denaturing conditions the latter protein was split up into two proteins with molecular masses of 14.5 kD (PR-r_1) and 13 kD (PR-r_2). Antibodies were raised to PR-R.

When antisera to P2 from tomato and PR-R from tobacco were used to probe western blots of PR proteins, both sera detected the same proteins (Fig. 6). On blots of low pH gels, both sera recognized the tomato P2 (Fig. 6, lanes 1 and 2), while on blots of high pH gels a band comigrating with PR-R was recognized (Fig. 6, lanes 3 and 4). On blots of SDS gels, tomato P2 was detected (Fig. 6, lanes 5 and 7) and in apoplastic fluid from tobacco two proteins with molecular masses of 14.5 kD and 13 kD were recognized by both antisera (Fig. 6, lanes 6 and 8).

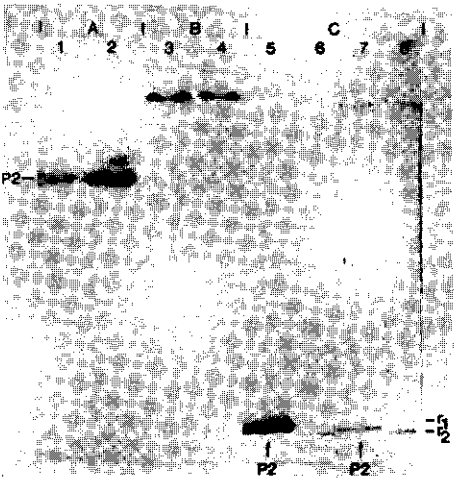

Figure 6. Immunoblots of PR proteins present in apoplastic fluids from *C. fulvum*-infected tomato electrophoresed on low pH, native gel (A, 200 μL, lanes 1 and 2) or SDS gel (C, 200 μL, lanes 5 and 7) and of PR proteins present in apoplastic fluids from TMV-infected tobacco electrophoresed on high pH, native gel (B, 50 μL, lanes 3 and 4) or SDS gel (C, 50 μL, lanes 6 and 8). The blots were probed with antiserum raised against P2 from tomato (lanes 1, 3, 5, and 6) or PR-R from tobacco (lanes 2, 4, 7, and 8).

DISCUSSION

A PSTV infection of tomato induces the synthesis of a protein which, after its apparent molecular mass of 14 kD, has been designated P14 (1, 2). The protein was purified and antibodies were raised (2). Using these antibodies, we previously detected three protein bands on blots of apoplastic proteins from *C. fulvum*-infected tomato electrophoresed on low pH, native gels (10; note that in this reference P2 and P4 were mislabeled as P3 and P5 as these latter two correspond to the 35-kD and 33-kD 1,3-β-glucanase, respectively, in apoplastic fluids from *C. fulvum*-infected tomato leaves [17]). Hence, we suggested that P2, P4, and P6 were three differently charged isomers of P14 (10).

Here we have described the purification and characterization of P2, P4, and P6. In contrast to what we expected, only two of the three proteins were found to be serologically related; whereas antibodies to P4 and P6 cross-reacted with P6 and P4 respectively, no serological relationship was detected between P2 and the other PR proteins from tomato. This lack of a serological relationship between P4 and P6 on the one hand, and P2 on the other can be explained by the impurity of the original preparation of P14 used to raise antibodies (2). To purify P14, Camacho Henriquez and Sänger (2) used cation exchange chromatography (one step elution) and anion exchange chromatography (the protein did not bind to the column). Since no salt gradient was used to elute the bound proteins from the cation exchange column, proteins with similar pI values were not separated. Indeed, isoelectric focusing of P14 revealed three components: a major one with a pI of 10.7, and two minor ones with pI values of 10.0 and 11.0, respectively (2). Probably the major component of P14 purified by Camacho Henriquez and Sänger corresponds to P6 in this paper; P6 is the most predominant of the three PR proteins and its pI is identical to the one of the major component of P14. The minor component with pI 11.0 could be identical to the serologically related P4 (pI 10.9), while the component with pI 10.0 may correspond to the unrelated P2 (pI 10.4 in our study).

Lucas and co-workers (21) have determined the amino acid sequence of P14; apparently the minor components did not interfere with the sequencing. Preliminary sequence data on P6 suggest that this protein is identical to the major component of P14 (data not shown). A further characterization of P4 and P6 will be accomplished by sequencing of the proteins and the isolation of cDNA and genomic clones.

The cross-reactivity of antibodies raised to P14 with the tobacco PR-1 family proteins found by Nassuth and Sänger (23) was confirmed by us. Both antisera raised against P4 or P6 cross-reacted with PR-1a, -1b, and -1c and antibodies raised against PR-1a, -1b, and -1c cross-reacted with P4 and P6, indicating that the tomato P4 and P6 and the tobacco PR-1a, -1b, and -1c proteins are serologically related. Antibodies raised to P14 cross-reacted with two other proteins (molecular mass 17 and 27.5 kD) on blots of tomato PR proteins separated under denaturing conditions (23). The antibodies were not checked on blots of low pH, native gels. With antiserum raised to P4 or P6 we did not find cross-reactions with proteins of higher molecular mass (results not shown).

Antibodies raised against P2 cross-reacted with PR-R from tobacco, a protein that migrates as two bands on SDS gels (r_1 and r_2, molecular mass 14.5 and 13 kD, respectively). The cross-reactivity of antibodies raised to P2 with both PR-r_1 and PR-r_2 suggests that these two tobacco proteins are related to each other as well. Antibodies raised against PR-R detected P2 on blots of tomato PR proteins electrophoresed under low pH, nondenaturing or denaturing conditions.

Recently, a possible biological function has been ascribed to some of the tomato PR proteins (11, 17, 28). Although there is no evidence for a possible role of P2, P4, and P6 in disease resistance, the early induction of these proteins in incompatible tomato-*C. fulvum* interactions justifies further research.

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CHAPTER 8

CHARACTERIZATION OF MESSENGER RNA FOR THE TOMATO PATHOGENESIS-RELATED PROTEIN P6, THE MOST ABUNDANT ISOMER OF P14

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ABSTRACT

P14 is one of the most predominant pathogenesis-related (PR) proteins that appears in the intercellular spaces of tomato (*Lycopersicon esculentum*) after infection by *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif). A cDNA clone containing a complete mRNA for P6, the most abundantly occurring isomer of P14, was isolated from cDNA libraries made with poly(A)-RNA isolated from *C. fulvum*-infected tomato leaves. The mRNA contains an open reading frame of 477 nucleotides, encoding a protein of 159 amino acids with a N-terminal signal peptide of 24 amino acids. By using the cDNA clone as a probe on a Northern blot containing poly(A)-RNA isolated from incompatible and compatible interactions at different times after inoculation, it was shown that the synthesis of P6 is regulated at the transcriptional level. In the incompatible interaction (Cf4/ race 5) there is a much faster accumulation of the P6 mRNA than in the compatible one (Cf5/ race 5). However, accumulation of P6 mRNA occurs transiently in the incompatible interaction, while there is a slow, but continuous, accumulation in the compatible one. Southern blot analysis revealed that there are at least two, but possibly four, genes present in the genome of tomato that encode P14-like proteins. Some of these genes might be pseudogenes.

INTRODUCTION

One of the most predominant pathogenesis-related (PR) proteins that appears in the intercellular spaces of tomato leaves upon infection by viroids, viruses or fungi is P14 (1, 8, 9, 10). The protein was purified from potato spindle tuber viroid-infected tomato leaves (2) and its amino acid sequence was determined (14). De Wit and Van der Meer (7) showed that in the interaction between *Cladosporium fulvum* and tomato, the accumulation of P14 in the apoplast could be used as an early marker of incompatibility. According to Vera *et al* (22, 23) the protein is associated with cell degeneration as a result of pathogenic attack.

We have shown that in apoplastic fluids that were obtained from *C. fulvum*-infected tomato, three basic PR proteins are present that migrate similarly to P14 on denaturing polyacrylamide gels (13). Purification and serological characterization of these proteins revealed that two of the proteins (P4 and P6, 15.5 kD, pI 10.9 and 10.7, respectively) were serologically related to each other and to the tobacco PR-1 proteins. P4 and P6 represent two differently charged isomers of P14. A third protein (P2, 15 kD, pI 10.4) was not serologically related to any other tomato PR protein, but was found to be related to PR-R (PR-4 according to the nomenclature of Van Loon (21)) from tobacco (13).

In this report we describe the molecular characterization of messenger RNA for P6, the most abundantly occurring isomer of P14 (13). An expression library, made with cDNA that was obtained from a compatible *C. fulvum*-tomato interaction, was screened with antiserum raised against P6 and a partial cDNA clone encoding the protein was obtained. Probing another cDNA library with this clone resulted in the identification of a full-length cDNA clone of P6. The clone was used to study the timing of accumulation of the P6 mRNA after infection by an avirulent or a virulent race of *C. fulvum* and to perform a Southern blot analysis of the P14 gene family.

MATERIALS AND METHODS

PLANTS, FUNGI AND INOCULATIONS

Near-isogenic lines of tomato cv Moneymaker were grown as described by De Wit and Flach (6) and inoculated with conidial suspensions of races of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) according to the method described by De Wit (5).

RNA ISOLATION AND NORTHERN BLOTTING

Leaf tissue was homogenized in guanidine-HCl buffer (8 M guanidine-HCl/20 mM MES/20 mM EDTA/50 mM ß-mercapto ethanol, pH 7) and extracted with phenol/chloroform (1:1) and chloroform. RNA was precipitated overnight with 2 M LiCl and poly(A)-RNA was obtained by affinity chromatography on oligo(dT)-cellulose. Poly(A)-RNA was electrophoresed on agarose gels containing formaldehyde and blotted onto Hybond N^+ membranes (Amersham) as described by Maniatis *et al* (15).

DNA ISOLATION AND SOUTHERN BLOTTING

DNA was isolated from freeze-dried leaf tissue of near-isogenic lines Cf4, Cf5 and Cf9, according to the CTAB nucleic acid extraction procedure (18). Approximately 5 μg of DNA of each of the cultivars was treated with RNAse and digested with either EcoRI or EcoRV in 500 μL end volume. The digested DNA was electrophoresed on 0.8% agarose gels, depurinated, denatured and blotted in 10x SSC onto Hybond N^+ with a vacuum blotter (Millipore).

HYBRIDIZATION OF BLOTS WITH DNA PROBES

DNA probes were random-primed labeled according to the oligolabeling procedure described by Hodgson and Fisk (11). Blots were hybridized in 5x SSC/5x Denhardt's/0.5% SDS/0.01% (w/v) salmon sperm DNA at 65°C and washed in 2x SSC/0.5% SDS or 0.5x SSC/0.5% SDS, at 65°C.

cDNA SYNTHESIS AND CONSTRUCTION OF LIBRARIES

For the first library, cDNA was synthesized on 5 μg of poly(A)-RNA, isolated from the interaction Cf5/race 5 at 14 days after inoculation, by using the Protoclone cDNA kit (Promega). A cDNA expression library in λgt11 (Promega), consisting of $1.1x10^5$ independant recombinants, was constructed as described by Van Kan *et al* (20). After amplification the library was stored at 4°C. A second library (consisting of $2x10^4$ independant recombinants) was made in the λZAP cloning vector (Stratagene) with cDNA that was synthesised on poly(A)-RNA isolated from the interaction Cf5/race 5 at 11 days after inoculation.

SCREENING OF cDNA EXPRESSION LIBRARY WITH ANTIBODIES RAISED AGAINST P6

Recombinant λgt11 phages and bacteria (*E. coli*, strain Y 1090 r$^-$) were combined to obtain $5x10^4$ plaque forming units, incubated at 37°C for 30 min and plated in top agar. After incubation at 42°C for 2 h, nitrocellulose filters (Schleicher and Schüll), soaked in 0.25% (w/v) IPTG and dried, were placed on the top agar and the plates were incubated at 37°C for 4 h. Filters were incubated with polyclonal antiserum raised against P6, diluted 1:500 in 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 0.1% (v/v) Tween 20, 0.5% (w/v) BSA and 1% (v/v) *E. coli* lysate. The antigen-antibody complexes were detected with the Bio-Rad Immun-Blot goat anti-rabbit alkaline phosphatase (GAR-AP) assay kit. Pure phages were obtained after a second screening.

SCREENING OF λZAP cDNA LIBRARY WITH A PARTIAL P6 cDNA CLONE

Hybond N$^+$ filters containing $2x10^4$ cDNA recombinant phages were screened with an oligolabeled cDNA probe of P6 that was obtained by screening the expression library with antibodies raised against P6. After hybridization (similar to Northern or Southern blots), filters were washed in 0.5x SSC/0.5% SDS, at 65°C. Phages were purified after a second screening and recombinant plasmids were obtained according to the manufacturer's protocol (Stratagene).

CLONING PROCEDURES AND DNA SEQUENCING

The various cloning procedures were performed according to Maniatis *et al* (15). Plasmid DNA was sequenced according to the chain termination method of Sanger *et al* (19), using [α-^{35}S]-dATP label and T3, T7 or SP6 promoter primers (Promega).

RESULTS

SCREENING OF cDNA EXPRESSION LIBRARY WITH ANTIBODIES RAISED AGAINST P6

Screening of filters containing a total of 10^5 plaques, with polyclonal antibodies raised against P6, resulted in the identification of 8 positive plaques. After a second screening pure phages were obtained and phage DNA was isolated from confluent plates. Seven phages were shown to contain a cDNA insert, ranging from 200 to 700 base pairs in length. The inserts of six phages were subcloned. Sequencing of the cDNA insert from the phage that gave the clearest positive signal upon screening with antibodies revealed a DNA sequence encoding a protein that was identical to 22 amino acids of the C-terminal part of P14 published by Lucas *et al* (14). The insert was isolated, labeled and hybridized to a blot containing the six subcloned inserts. Two larger inserts were found to cross-hybridize with the probe. The largest insert was shown to contain a partial cDNA clone of P14 with a coding region of 180 base pairs. The corresponding amino acid sequence was identical to amino acids 76 (Pro) to 130 (Tyr) of the sequence published by Lucas *et al* (14). However, in the sequence determined by us five additional amino acids (Trp-Arg-Asn-Ser-Val) were present between amino acids 98 (Val) and 99 (Arg) of the published sequence of P14. Comparison of this additional amino acid sequence block with the sequences of tobacco PR proteins PR-1a, -1b and -1c (3, 16), revealed that it is also present in these proteins, indicating that Lucas and co-workers (14) had overlooked this stretch of amino acids after cleavage of the protein.

SCREENING OF λZAP LIBRARY WITH A PARTIAL cDNA CLONE OF P6

To isolate a full-length cDNA clone of P6, a primary λZAP library, consisting of $2x10^4$ independant recombinants, was screened with the longest partial P6 cDNA clone that was obtained from the λgt11 library. About 200 plaques (1%) hybridized with the probe. Thirty phages were purified in a second and third screening round and were shown to contain inserts ranging from 250 to 800 base pairs, with a majority greater than 500 base pairs. Performing only the ddT sequencing reaction on 24 of the cDNA inserts from the 3'-end, revealed that all clones had identical sequencing patterns, although several polyadenylation sites were observed. Therefore we presumed that all cDNA clones analysed originated from the same type of mRNA.

Three clones containing the longest inserts were sequenced and one clone was found to contain the complete open reading frame encoding P6. The complete nucleotide sequence of this P6 mRNA and the amino acid sequence of its translation product is presented in Figure 1. The open reading frame of 477 nucleotides encodes a precursor protein of 159 amino acids, of which the first 24 amino acids represent the signal peptide. Apart from the additional five amino acids, the amino acid sequence of the mature P6 protein is identical to the sequence of P14, published by Lucas *et al* (14). Based on our data the molecular weight of the mature P6 was calculated to be 14,843 daltons.

TIME COURSE OF ACCUMULATION OF P6 mRNA

Poly(A)-RNAs, isolated from leaflets obtained from the interactions Cf4/race 5 (incompatible) and Cf5/race 5 (compatible) at 4, 6, 8, 10, 12 and 14 days after inoculation, were electrophoresed and blotted. Poly(A)-RNAs isolated from uninoculated Cf5 plants and from race 5 of *C. fulvum* grown *in vitro* were included as controls. The blot was hybridized with the largest P6 cDNA insert that was obtained from the expression library. The P6 mRNA was not detected in healthy plants and the probe did not cross-react with a fungal mRNA (Fig. 2). In the incompatible interaction accumulation of P6 mRNA was transient; the mRNA was already present at 4 days after inoculation and increased in concentration up to 8 days after inoculation. After this period the amount of P6 mRNA decreased. In the compatible interaction the mRNA started to accumulate

at 6 days after inoculation and reached a higher concentration than in the incompatible interaction. At 14 days after inoculation also in the compatible interaction the amount of P6 mRNA had decreased to some extent.

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AAAATGGGGTTGTTCAACATCTCATTGTTACTCACTTGTCTCATGGTATTAGCCATATTT   60
   MetGlyLeuPheAsnIleSerLeuLeuLeuThrCysLeuMetValLeuAlaIlePhe
                  ↓
CACTCTTGTGAGGCCCAAAATTCACCCCAAGACTATCTTGCGGTTCATAACGATGCCCGT  120
HisSerCysGluAlaGlnAsnSerProGlnAspTyrLeuAlaValHisAsnAspAlaArg

GCCCAAGTCGGAGTCGGGCCTATGTCTTGGGATGCCAACTTGGCATCCCGAGCACAAAAC  180
AlaGlnValGlyValGlyProMetSerTrpAspAlaAsnLeuAlaSerArgAlaGlnAsn

TATGCCAACTCAAGAGCTGGTGATTGTAACTTGATTCATTCTGGTGCTGGGGAGAATCTT  240
TyrAlaAsnSerArgAlaGlyAspCysAsnLeuIleHisSerGlyAlaGlyGluAsnLeu

GCCAAGGGTGGTGGTGACTTCACGGGGAGGGCAGCCGTGCAATTGTGGGTGTCCGAGAGG  300
AlaLysGlyGlyGlyAspPheThrGlyArgAlaAlaValGlnLeuTrpValSerGluArg

CCAAGCTATAACTACGCTACCAACCAATGTGTTGGTGGAAAAAAGTGTAGACATTATACT  360
ProSerTyrAsnTyrAlaThrAsnGlnCysValGlyGlyLysLysCysArgHisTyrThr

CAAGTAGTCTGGCGCAACTCAGTCCGACTAGGTTGTGGTCGGGCACGTTGCAACAACGGA  420
GlnValValTrpArgAsnSerValArgLeuGlyCysGlyArgAlaArgCysAsnAsnGly

TGGTGGTTCATTTCTTGCAACTATGATCCTGTAGGCAACTGGATCGGACAACGTCCTTAC  480
TrpTrpPheIleSerCysAsnTyrAspProValGlyAsnTrpIleGlyGlnArgProTyr

TAAAATGATGTATACTTATGACATGTTGCTAGTATTAAATAAAATTCTCATATGAGACGT  540
End

                                                          ▼
CGAGAAGTTAAAATTTAAGTTTGACATATGAATCAAGTCAAACTCCTATCTAAAATATTA  600

    ▼       ▼       ▼       ▼               ▼
AGGGATTAAATATTGAACATCTATAATTATTATTATTTCCCTTTTGATGTTGCTAATATG  660

          ▼   ▼       ▼
AATAATTCCACATACCATATGTTCATAATGGGCTTAAGTTGATTATTAAGTACTGCATCT  720

                                    ▼
TCTTGTTTCCATAAAACATTAATATACATAAAATTTTAATTA    763
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Figure 1. Nucleotide and corresponding amino acid sequence of P6 cDNA. The arrow indicates the cleavage site between the signal peptide and the mature P6 protein. The five additional amino acids, overlooked by Lucas *et al* (14), are underlined. Closed triangles (▼) indicate the different polyadenylation sites that were observed in other P6 cDNA clones.

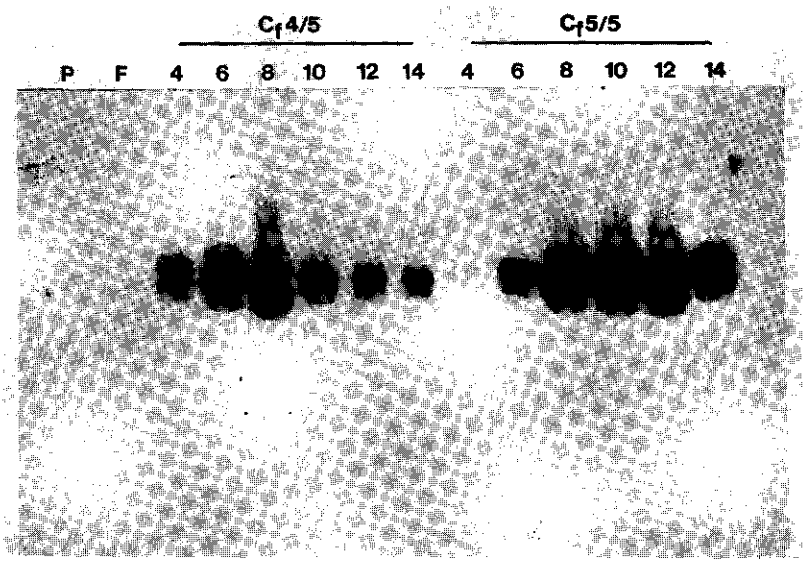

Figure 2. Time course of accumulation of P6 mRNA. Poly(A)-RNA was isolated from non-inoculated near-isogenic line Cf5 (lane marked 'P'), *C. fulvum* (race 5) grown *in vitro* (lane marked 'F') and from an incompatible (Cf4/race 5) and a compatible (Cf5/race 5) *C. fulvum*-tomato interaction, at 4, 6, 8, 10, 12 and 14 days after inoculation. The Northern blot was hybridized (at 65°C) with a labeled, partial cDNA clone of P6 and washed in 2x SSC/0.5% SDS, at 65°C.

SOUTHERN BLOT ANALYSIS OF THE GENES ENCODING P14

DNA, isolated from leaves of near-isogenic lines Cf4, Cf5 or Cf9, was digested with EcoRI or EcoRV, electrophoresed and blotted. Hybridization of the blot with a labeled insert that contained a complete copy of P6 mRNA, revealed fragments of approximately 5.5 kb in length that hybridized strongly (Fig. 3). The presence of two strongly hybridizing and one to two weakly hybridizing bands in all digests suggests that there are two, but possibly three or four genes present that code for P14. Some of these genes might be pseudogenes. Between the three cultivars no polymorphism was detected.

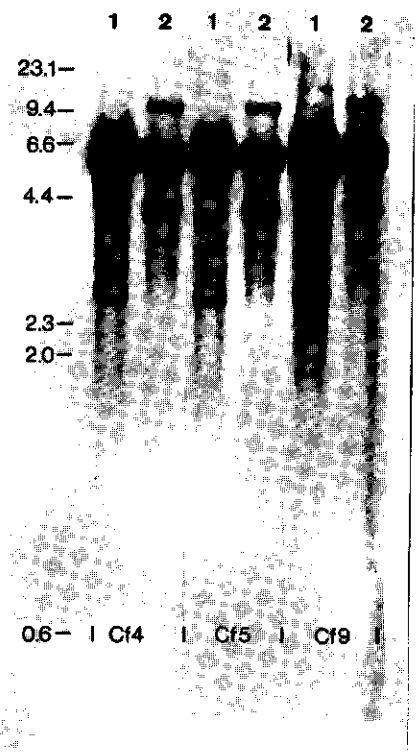

Figure 3. Southern blot analysis of total DNA isolated from non-inoculated near-isogenic lines Cf4, Cf5 or Cf9. 5μg of DNA was digested with either EcoRI (lanes 1) or EcoRV (lanes 2), electrophoresed and blotted. The blot was hybridized (at 65°C) with a labeled insert that contained a complete copy of P6 mRNA and washed in 0.5x SSC/0.5% SDS, at 65°C.

DISCUSSION

A full length cDNA clone of P6, the most abundantly occurring isomer of the tomato PR protein P14, was isolated by screening a λgt11 cDNA library with antibodies raised against P6 and using the partial cDNA clone that was obtained as a probe to screen a λZAP cDNA library. The amino acid sequence analysis

of P14 that was performed by Lucas *et al* (14) was shown to be incomplete; five amino acids, also present in PR-1a, -1b and -1c of tobacco (3, 16), were overlooked. With addition of these five amino acids, the mature P6 protein is 61% identical and 74% similar to PR-1a of tobacco.

The translation product of the mRNA of P6 contains a signal peptide of 24 amino acids at the NH_2-terminus of the mature protein. The exact cleavage site can be deduced from the protein sequence data of Lucas *et al* (14). The signal peptide meets all the criteria observed for eukaryotic signal peptides (25) and is similar to the signal peptides of the tobacco PR-1 family (3, 16). It is removed after targeting of the precursor peptide to the endoplasmatic reticulum (24). This supports the results of Vera *et al* (23), who showed that in cell-free translation systems P14 is synthesized as a 16.5 kD precursor protein.

The synthesis of P6 is regulated at the transcriptional level. In the incompatible *C. fulvum*-tomato interaction Cf4/race 5 accumulation of P6 mRNA is transient and occurs much faster than in the compatible interaction Cf5/race 5. These observations correspond to the time course of accumulation of the protein in apoplastic fluids isolated from incompatible or compatible interactions between tomato and *C. fulvum* (7, 8, 12).

The presence of two isomers of P14 suggests that in tomato at least two functional genes are present that encode these proteins. According to the Southern blot analysis there are at least two, but possibly four genes present, some of which might be pseudogenes. In the tobacco genome there are at least 8 genes present that encode the acidic PR-1 proteins (4, 17). However, only three of these genes are expressed, indicating that the other five are probably pseudogenes. The tobacco PR-1 proteins have a basic isoform that shows approximately 65% amino acid similarity to the acidic PR-1 proteins (4). We have not observed an acidic isoform of the basic P14 proteins in *C. fulvum*-infected tomato leaves (13).

The availability of a complete P6 cDNA clone might enable us to isolate a cDNA clone representing P4, the less abundant occurring isomer of P14. The P6 cDNA clone can also be used as a probe to isolate the gene encoding P6, with its regulatory elements, from a genomic library of tomato. In order to learn which of the regulatory elements are important for the induction, tomato plants can be transformed with these regulatory elements fused to the GUS reporter gene and subsequently can be tested for GUS activity after inoculation with

virulent or avirulent races of *C. fulvum*. Alternatively plants can be transformed with the clone linked to a constitutive plant promotor and can subsequently be tested for their susceptibility to *C. fulvum*. These experiments might provide more insight in a possible role of P6 in active plant defense.

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SUMMARY

In this thesis research on the physiological and molecular aspects of pathogenesis in the interaction between tomato and *Cladosporium fulvum* Cooke (syn. *Fulvia fulva* [Cooke] Cif) is described. This plant-fungus interaction is envisaged to be based on a gene-for-gene relationship. Incompatible interactions (plants are resistant) between certain races of *C. fulvum* and tomato are thought to result from a specific interaction between products of fungal avirulence genes (race-specific elicitors) and products of corresponding resistance genes (cultivar-specific receptors) that are present in the host. After the elicitor has bound to the receptor, host defense genes are activated. A major feature of the activation of host defense is the accumulation of several pathogenesis-related (PR) proteins. Generally these proteins, which also accumulate in several other plant species, are of low molecular weight, accumulate in the apoplast, are highly resistant to proteolytic cleavage and have extreme iso-electric points. In compatible interactions (plants are susceptible) presumably no molecular recognition of the fungus occurs, resulting in colonization of the apoplastic space between the leaf mesophyll cells.

In chapter 2 the purification of a fungal protein (designated P1, molecular mass 14 kD), specific for compatible *C. fulvum*-tomato interactions is described. Polyclonal antibodies were raised and the protein was shown to be only present in apoplastic fluid isolated from compatible *C. fulvum*-tomato interactions. Immunolocalization experiments revealed that in compatible interactions the protein was present in the electron-dense matrix between the walls of leaf mesophyll cells and fungal hyphae (chapter 6). Probably P1 plays a role in the establishment or maintenance of basic compatibility and can be regarded as a basic pathogenicity factor.

In compatible interactions the fungus is able to hydrolyze the translocation sugar sucrose to glucose and fructose, which in turn are converted into the polyol mannitol by mannitol dehydrogenase (MTLDH) (chapter 3). During the colonization process of the intercellular spaces of the tomato leaves, increasing amounts of mannitol present in the apoplastic fluid coincided with increasing levels of MTLDH activity. The fungal metabolite mannitol cannot be utilized by

the plant and possibly functions as a carbohydrate reserve for the fungus. In incompatible interactions no functional nutritional relationship between host and fungus is established and consequently no mannitol accumulation was observed.

Chapter 4 describes the partial purification of a race-specific elicitor, the putative product of avirulence gene 4 (*avr4*) of *C. fulvum*. The race-specific elicitor precipitated in 60% (v/v) acetone, migrated on high pH, native gels and bound to an anion-exchange column at pH 9.0. The elicitor preparation induced a hypersensitive response and accumulation of PR proteins in near-isogenic line Cf4 of tomato (carrying resistance gene 4), indicating that active host defense is triggered by recognition of a race-specific elicitor by the plant.

In incompatible interactions between tomato and *C. fulvum* the inhibition of fungal growth coincides with a substantial accumulation of PR proteins in the apoplast of the tomato leaf (chapter 5). Two abundantly occurring PR proteins of 35 kD and 26 kD in molecular mass were purified and were shown to have 1,3-ß-glucanase and chitinase activity, respectively. Fungal walls that partly consist of 1,3-ß-glucans and chitin, can be affected by these hydrolytic enzymes. With polyclonal antibodies that were raised against the purified enzymes one additional 1,3-ß-glucanase (33 kD) and three additional chitinases (27, 30 and 32 kD) were detected in apoplastic fluids or homogenates of tomato leaves after inoculation with *C. fulvum*. Upon inoculation with *C. fulvum* apoplastic chitinase and 1,3-ß-glucanase activities increased more rapidly in incompatible interactions than in compatible ones, indicating that these hydrolytic enzymes might play a role in active host defense.

Immunolocalization experiments revealed that in incompatible tomato-*C. fulvum* interactions 1,3-ß-glucanases and chitinases accumulated in intercellular spaces, cytoplasm and electron-dense material that was present in the vacuoles of leaf mesophyll cells (chapter 6). Often 1,3-ß-glucanases and chitinases were found to be associated with the electron-dense outer layer of the fungal cell wall. In compatible interactions no localized accumulation of 1,3-ß-glucanases and chitinases was observed.

In addition to the rapid induction and accumulation of 1,3-ß-glucanases and chitinases in incompatible tomato-*C. fulvum* interactions, a substantial accumulation of PR proteins of about 15 kD in molecular mass occurred. It was shown that in apoplastic fluids isolated from induced tomato leaves three basic PR proteins are present that migrate similarly to the earlier characterized tomato

PR protein P14 on SDS-polyacrylamide gels (chapter 7). Two proteins, designated P4 and P6, molecular mass 15.5 kD, isoelectric points (pI) 10.9 and 10.7, respectively, appeared to be serologically related to each other and to the tobacco PR-1 proteins. The third protein, designated P2, molecular mass 15 kD, pI 10.4, was found to be serologically related to PR-R from tobacco. The biological function of P2, P4 and P6 is still unknown.

In chapter 8 the characterization of messenger RNA (mRNA) for P6, the most abundant isomer of P14, is described. The mRNA contains an open reading frame of 477 nucleotides, encoding a protein of 159 amino acids, with an N-terminal signal peptide of 24 amino acids. Synthesis of P6 is regulated at the transcriptional level. In the incompatible interaction Cf4/race 5 there was a much faster accumulation of the P6 mRNA than in the compatible one (Cf5/race 5). There are probably two to four genes present in the genome of tomato that encode P14-like proteins.

SAMENVATTING

Dit proefschrift beschrijft onderzoek naar de fysiologische en moleculaire aspecten van de pathogenese in de interactie tussen tomaat en *Cladosporium fulvum* Cooke (syn. *Fulvia fulva* [Cooke] Cif). Deze plant-schimmel interactie wordt verondersteld gebaseerd te zijn op een gen-om-gen relatie. Incompatibele interacties (planten zijn resistent) tussen bepaalde fysiologische rassen van *C. fulvum* en tomaat zijn waarschijnlijk het resultaat van een specifieke interactie tussen producten van avirulentiegenen van de schimmel (fysio-specifieke elicitoren) en producten gecodeerd door corresponderende resistentiegenen (cultivar-specifieke receptoren) die aanwezig zijn in de gastheer. Na interactie van de elicitor met de receptor worden de afweergenen van de gastheer geactiveerd. Een belangrijk gevolg van deze activering is de accumulatie van verschillende pathogenese-gerelateerde (PR) eiwitten. In het algemeen hebben deze eiwitten, die ook in verschillende andere plantesoorten voorkomen, een laag molecuulgewicht, en accumuleren ze in de apoplast, zijn weinig gevoelig voor proteolytische splitsing en hebben een extreem isoelectrisch punt. In compatibele interacties (planten zijn vatbaar) wordt de schimmel waarschijnlijk niet herkend door de waardplant en vindt kolonisering van de apoplastische ruimte tussen de mesofylcellen van het blad plaats.

In hoofdstuk 2 wordt de zuivering beschreven van een eiwit afkomstig van de schimmel (P1 genaamd, molecuulgewicht 14 kD), dat specifiek is voor compatibele interacties tussen *C. fulvum* en tomaat. Er werden polyklonale antilichamen opgewekt en er werd aangetoond dat het eiwit enkel aanwezig was in apoplastische vloeistof geïsoleerd uit compatibele *C. fulvum*-tomaat interacties. Immunolocalisatie experimenten toonden aan dat in compatibele interacties het eiwit aanwezig is in de electronendichte matrix tussen de wanden van mesofylcellen van het blad en schimmelhyfen (hoofdstuk 6). Waarschijnlijk speelt P1 een rol bij het verkrijgen of in stand houden van basiscompatibiliteit en kan worden beschouwd als een basale pathogeniciteitsfactor.

In compatibele interacties is de schimmel in staat de transportsuiker sucrose te hydrolyseren tot glucose en fructose en deze vervolgens om te zetten in de polyol mannitol onder invloed van mannitoldehydrogenase (MTLDH)

(hoofdstuk 3). Tijdens de kolonisering van de intercellulaire ruimten van de tomatebladeren kon in intercellulaire vloeistof een positieve correlatie gevonden worden tussen de hoeveelheid geaccumuleerd mannitol en de hoogte van de MTLDH activiteit. De schimmelmetaboliet mannitol kan niet door de plant worden omgezet en functioneert waarschijnlijk als een koolhydraatreserve voor de schimmel. In incompatibele interacties ontstaat er geen functionele voedingsrelatie tussen plant en schimmel en vond derhalve geen ophoping van mannitol plaats.

Hoofdstuk 4 behandelt de gedeeltelijke zuivering van een fysio-specifieke elicitor; het veronderstelde product van avirulentiegen 4 (*avr4*) van *C. fulvum*. De fysio-specifieke elicitor precipiteerde in 60% (v/v) aceton, migreerde op natieve, hoge pH gels en bond aan een anionenwisselaar bij pH 9.0. Het elicitor preparaat induceerde een overgevoeligheidsreactie en de accumulatie van PR eiwitten in bijna-isogene lijn Cf4 van tomaat (bevat het resistentiegen 4). Dit impliceert dat actieve afweer bij de gastheer wordt geïnduceerd door herkenning van een fysio-specifieke elicitor door de plant.

In incompatibele interacties tussen tomaat en *C. fulvum* gaat remming van de schimmelgroei gepaard met een sterke accumulatie van PR eiwitten in de apoplast van het tomateblad (hoofdstuk 5). Twee PR eiwitten die in grote hoeveelheid voorkomen en een molecuulgewicht bezitten van 35 kD en 26 kD, werden gezuiverd en bleken respectievelijk 1,3-ß-glucanase en chitinase activiteit te bezitten. Celwanden van schimmels die gedeeltelijk uit 1,3-ß-glucanen en chitine bestaan, kunnen door deze hydrolytische enzymen worden aangetast. Met behulp van polyklonale antilichamen die tegen de gezuiverde enzymen werden opgewekt, werden in apoplastische vloeistoffen of homogenaten afkomstig van geïnoculeerde tomatebladeren nog één andere 1,3-ß-glucanase (33 kD) en drie andere chitinases (27, 30 en 32 kD) gedetecteerd. Na inoculatie met *C. fulvum* steeg de apoplastische chitinase- en 1,3-ß-glucanase activiteit sneller in incompatibele interacties dan in compatibele, zodat de mogelijkheid bestaat dat deze hydrolytische enzymen een rol spelen bij de actieve afweer van de gastheer.

Immunolocalisatie experimenten toonden aan dat in incompatibele tomaat-*C. fulvum* interacties accumulatie van 1,3-ß-glucanases en chitinases plaatsvond in intercellulaire ruimtes, cytoplasma en electronen-dicht materiaal dat aanwezig was in de vacuolen van de mesofylcellen van het blad (hoofdstuk 6). Vaak waren de 1,3-ß-glucanases en chitinases aanwezig op de electronen-dichte buitenlaag van

de schimmelcelwand. In compatibele interacties kon geen gelocaliseerde accumulatie van 1,3-ß-glucanases en chitinases worden aangetoond.

Naast de snelle inductie en accumulatie van 1,3-ß-glucanases en chitinases, vond er in incompatibele interacties een sterke ophoping van PR eiwitten met een molecuulgewicht van ongeveer 15 kD plaats. Er werd aangetoond dat in apoplastische vloeistof geïsoleerd uit geïnduceerde tomatebladeren, drie basische PR eiwitten aanwezig zijn die eenzelfde migratie vertonen op SDS-polyacrylamide gels als het eerder gekarakteriseerde PR eiwit P14 (hoofdstuk 7). Twee eiwitten, genaamd P4 en P6, met een molecuulgewicht van 15,5 kD en isoelectrische punten (pI) van respectievelijk 10,9 en 10,7, bleken serologisch verwant te zijn aan elkaar en aan de PR-1 eiwitten van tabak. Het derde eiwit, genaamd P2, met een molecuulgewicht van 15 kD en pI van 10,4, was serologisch verwant aan PR-R van tabak. De biologische functie van P2, P4 en P6 is nog onbekend.

In hoofdstuk 8 wordt de karakterisering van messenger RNA (mRNA) voor P6, het meest voorkomende isomeer van P14, beschreven. Het mRNA bevat een open leesraam van 477 nucleotiden, coderend voor een eiwit van 159 aminozuren met een N-terminaal signaalpeptide van 24 aminozuren. De synthese van P6 wordt op transcriptieniveau gereguleerd. In de incompatibele interactie Cf4/fysio 5 accumuleert het P6 mRNA veel sneller dan in de compatibele interactie (Cf5/fysio 5). Waarschijnlijk zijn er twee tot vier genen aanwezig in het tomategenoom die coderen voor eiwitten die homologie vertonen met P14.

CURRICULUM VITAE

Matthieu Henri Antoon Jozef Joosten werd op 28 april 1962 te Hilversum geboren. In 1980 behaalde hij het diploma Atheneum B aan de scholengemeenschap 'Laar en Berg' te Laren (N.H.), waarna de studie werd voortgezet aan de Landbouwuniversiteit te Wageningen. In 1986 werd het ingenieursexamen Tuinbouwplantenteelt afgelegd, met als hoofdvakken Tuinbouwplantenteelt en Fytopathologie en als bijvakken Plantenfysiologie en Organische chemie. Na het afronden van zijn afstudeeropdracht bij dr. ir. P.J.G.M. de Wit van de vakgroep Fytopathologie, was hij als toegevoegd onderzoeker bij deze vakgroep werkzaam van oktober 1986 tot april 1990. De resultaten van het gedurende deze periode uitgevoerde onderzoek zijn beschreven in dit proefschrift. Sinds december 1990 is hij werkzaam als post-doctoraal onderzoeker bij de vakgroep Fytopathologie van de Landbouwuniversiteit.