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Subcellular localization of fungal and plant proteins in the *Cladosporium fulvum*-tomato interaction

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Proefschrift

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Stellingen

1. Extracellulaire zure chitinases en 1,3-ß-glucanases uit tomaat spelen geen aantoonbare rol in de resistentie van tomaat tegen *Cladosporium fulvum*.
   Dit proefschrift.
   Joosten et al. 1994. Physiol. Mol. Plant Pathol. in press.

2. De betekenis van chitinases en 1,3-ß-glucanases in de resistentie van planten tegen schimmels mag niet gebaseerd worden op de resultaten verkregen met een isozym.
   Schröder et al. 1992. Plant J. 5: 57-63.

3. Het verhitten van intercellulaire vloeistof verkregen van *Cladosporium fulvum*-geïnfecteerde tomatebladeren, voorafgaande aan de bepaling van de necrose-inducerende werking van deze vloeistoffen gaat er vanuit dat alle necrose-inducerende elicitors van *C. fulvum* hittebestendig zijn, hetgeen nog niet is bewezen.
   Hammond-Kosack and Jones 1994. Mol. Plant-Microbe Interact. 7: 58-70.

4. Het voorkomen van twee P14-achtige eiwitten in schimmel-geïnfecteerde tomatewortels, wordt niet veroorzaakt door gedeeltelijke afbraak van P14.
   Benhamou et al. 1991. Physiol. Mol. Plant Pathol. 38: 237-253.

5. De bewering dat avenacosiden geen rol kunnen spelen in resistentie van haverplanten tegen obligate biotrofe schimmels is niet juist.
   Nisius 1988. Planta 173: 474-481.

6. Het gebruik van de term compatibel ter aanduiding van het vermogen van een pathogeen een plant te kunnen infecteren is verwarrend en niet juist.
   Jakobek et al. 1993. Plant Cell 5: 57-63.

7. Schimmelpathogeniteit is niet voor één gat te vangen.

8. Een gesloten teeltsysteem in de tuinbouw is niet duurzaam.

9. Files verplaatsen zich bij een verbeterde infrastructuur en verdwijnen bij verminderd autogebruik.

10. Wederzijdse waardering tussen sporter en scheidsrechter zal toenemen als ze vaker in elkaars schoenen staan.

Stellingen behorende bij het proefschrift
"Subcellular localization of fungal and plant proteins in the *Cladosporium fulvum*-tomato interaction".
Wageningen, 4 november 1994, J.P. Wubben

Content

chapter 1

General introduction and outline of this thesis

adapted from "Molecular and biochemical aspects
of host-pathogen interactions"
Jos P. Wubben, Thomas Boller, Guy Honée and Pierre J.G.M. de Wit
in *Resistance of crop plants against fungi*
(Hartleb, Heitefuss and Hoppe eds.) Fisher Verlag, Jena, in preparation

1. INTRODUCTION

Successful infection of plants by pathogenic fungi involves many different phases. The primary phase involves contact between plant and pathogen comprising adhesion and surface recognition. This phase can be followed by penetration which may require hydrolytic degradation of host cell walls. Establishment and maintenance within the host species are further requirements for successful infection. These latter phases may depend on different factors, such as production of phytotoxins, production of enzymes which can detoxify antimicrobial metabolites produced by plants, and production of suppressors or inactivators of host defence responses. The occurrence and importance of different pathogenicity determinants may differ between pathogens. These involve suppressing and inactivating host defence responses by the fungus. In addition, a nutritional relation between host plant and fungus should be established for some time. In resistant plants, specific recognition of the invading fungus may occur, leading to activation of defence responses, preventing further growth of the pathogen in the plant.

During the last two decades, many new techniques have been developed facilitating the study of infection processes at the molecular level. A few examples will be given, describing pathogenicity factors involved in different aspects of infection processes, which have been characterized in detail at the molecular level. Furthermore, new strategies for detailed molecular characterization of plant-pathogen interactions will be discussed. The interaction between the pathogenic fungus *Cladosporium fulvum* and tomato will be described in greatest detail, with emphasis on putative pathogenicity factors, race-specific elicitors, and resistance gene-dependent defence responses. Finally, the aim and outline of this thesis are presented.

2. MOLECULAR CHARACTERIZATION OF PATHOGENICITY FACTORS WITH KNOWN FUNCTIONS

2.1. The importance of cutinolytic enzymes

The first barrier fungal pathogens may encounter before entering their host plant is the cuticle, which consists of a structural polymer (cutin) embedded in wax. Degradation of the cuticle by cutin degrading enzymes was thought to be an essential step in early infection processes of pathogenic fungi which have to pass the plant cuticle in order to infect (recently reviewed by Köller, 1991). Experimental data provided evidence for a functional role of cutinase in penetration of the cuticle of pea plants by the fungus *Nectria haematococca* f.sp. *pisi* (Dickman *et al.*, 1989; Köller *et al.*, 1982; Maiti and Kolattukudy, 1979). However, requirement of cutinase has recently been disputed for this pathogen (Stahl and Schäfer, 1992). Disruption of the cutinase gene in *N. haematococca* resulted in a cutinase deficient

mutant which remained pathogenic on pea, indicating that cutinase is not required for pathogenicity in this plant-pathogen interaction (Stahl and Schäfer, 1992). However, more recently, detailed analyses of the *N. heamatoccoca* cutinase mutant did reveal a reduced virulence of this fungus on its host (Rogers *et al.*, 1994) indicating that cutinase indeed plays a role in the pathogenicity of this fungus.

2.2. Production and detoxification of host-selective toxins; HC-toxin of *Cochliobolus carbonum*

Host-specificity of fungal pathogens might be determined by host-selective toxins (HST) (reviewed by Walton and Panaccione, 1993). HSTs are known only for fungal pathogens, especially the genera of *Alternaria* and *Cochliobolus*. The known HSTs are in majority low molecular weight secondary metabolites. They are involved in aggressiveness and pathogenicity; a fungus that produces a HST causes more disease on its host compared to non-producing strains of the same species. Insensitivity of a plant to HST confers increased resistance to the producing organism.

Specificity of the interaction between *Cochliobolus carbonum* race 1 and maize is mediated by a cyclic tetrapeptide, HC-toxin. HC-toxin-biosynthetic enzymes and their encoding genes from *C. carbonum* have been isolated and characterized (Panaccione *et al.*, 1992; Walton and Holden, 1988). The central enzyme, HC-toxin synthase (HTS), is a 570-kD tetrapartite cyclic peptide synthetase (Walton and Holden, 1988), encoded by a 15.7-kB open-reading frame (ORF) called *HTS1* (Panaccione *et al.*, 1992). *HTS1* is present in two copies in all HC-toxin producing (tox^+) isolates examined, but is completely lacking in non-producing isolates (tox^-). Disruptions in both *HTS1* ORFs resulted in mutants of *C. carbonum* which were unable to develop disease symptoms on maize (Panaccione *et al.*, 1992). It is suggested that HC-toxin specifically suppresses the induction of genes that control active defence responses and thereby functions in pathogenicity.

Conversely, the gene in maize which confers resistance to *C. carbonum* race 1 (*Hm1*), has been cloned by transposon mutagenesis (Johal and Briggs, 1992). *Hm1* encodes the HC-Toxin Reductase (HCTR) which catalyses reduction of the 8-carbonyl group of the Aeo-residue (2-amino-9,10-epoxy-8-oxo-decanoic acid) of the tetrapeptide, which leads to inactivation of the toxin (Meeley *et al.*, 1992). Host-specificity in this interaction is, therefore, determined both at the plant level as well as at the pathogen level: for disease development, the fungus needs HC-toxin and to become resistant to the disease the plant needs the *Hm1* resistance gene which encodes a detoxifying enzyme.

2.3. Detoxification of antimicrobial compounds

Several low molecular weight antimicrobial compounds have been regarded as possible means by which plants defend themselves against fungal pathogens. These compounds may either

occur constitutive or be synthesized in response to microbial infection. For successful infection, detoxification of plant antimicrobial compounds might be a necessity for the fungus.

2.3.1. Detoxification of the preformed antifungal compound avenacin

Oat plants produce different antifungal saponins, one of which is avenacin, present in oat roots (Crombie *et al.*, 1986). Specificity of the oat pathogen *Gaeumannomyces graminis* f.sp. *avenae*, is supposed to depend on its ability to degrade avenacin (Osbourn *et al.*, 1991). A correlation has been found between production of an avenacin hydrolysing enzyme (avenacinase) by *G. graminis* isolates, and ability of the fungus to infect oats (Osbourn *et al.*, 1991). Transformation-mediated disruption of the avenacinase gene in *G. graminis* f. sp. *avenae* resulted in avenacinase$^-$ mutants which had become non-pathogenic on oats (Bowyer *et al.*, 1994). From these results, it can be concluded that avenacinase is an important host-specificity determinant which enables *G. graminis* f.sp. *avenae* to infect oat plants.

2.3.2. Detoxification of the induced antimicrobial compound pisatin

Phytoalexins are low molecular weight antimicrobial compounds which are hardly detectable in healthy plant tissue but are synthesized in response to microbial infection (reviewed by VanEtten *et al.*, 1989). Fungal pathogenicity may depend on detoxification of its hosts phytoalexins. Genetic analyses on the interaction between *N. haematococca* mating population VI and pea, had revealed a strong correlation between the ability of the fungus to demethylate the phytoalexin pisatin (Pda$^+$) and its pathogenicity on pea (VanEtten *et al.*, 1989). In addition, transformation of a *PDA* gene from *N. haematococca* to the maize pathogen *Cochliobolus heterostrophus* increased the size of lesions produced by this fungus on pea (Schäfer *et al.*, 1989). However, it has recently been demonstrated that Pda$^-$ mutants created by transformation-mediated gene-disruption, although showing some reduction in virulence, are still more pathogenic than naturally occurring Pda$^-$ isolates (VanEtten *et al.*, 1993). It has been shown before that *PDA* genes are located on a dispensable (DS) chromosome (Miao *et al.*, 1991). The disruption of a *PDA* gene now revealed that, in addition to the *PDA* gene other genes encoding pathogenicity factors are located on the DS chromosome. All naturally occurring Pda$^-$ isolates lack the DS chromosome (Miao *et al.*, 1991; VanEtten *et al.*, 1993).

3. NEW STRATEGIES TO CHARACTERIZE PUTATIVE PATHOGENICITY FACTORS

Recently, new molecular strategies have been employed to isolate factors possibly involved in pathogenicity of plant pathogenic fungi (Pieterse *et al.*, 1993b; Talbot *et al.*, 1993). It was

shown that a number of fungal genes, possibly involved in the infection process, was induced specifically *in planta*. Several strategies have been designed to dissect the infection process at the molecular level, based on induced expression of genes involved in the infection process, as has been shown before (Joosten *et al.*, 1994a; Van den Ackerveken *et al.*, 1993; VanEtten *et al.*, 1989; Van Kan *et al.*, 1991). These strategies include differential screening for isolation of *in planta* induced genes of fungal origin. In addition, random or insertional mutagenesis by transformation can be applied to isolate genes involved in the infection process. These mutations might either result in known phenotypes or in characteristic phenotypes which had not been observed before and which are caused by mutations in yet unknown genes. Characterization of the latter genes enables molecular dissection of new steps in the infection process. Two examples of genes discovered by the differential screening approach will be described.

3.1. *In planta* induced genes of the potato pathogen *Phytophthora infestans*

Differential hybridization of a genomic library of the pathogenic fungus *Phytophthora infestans* was used for selection and isolation of genes encoding proteins possibly involved in the infection process of *P. infestans* on potato (Pieterse *et al.*, 1993b). cDNA probes used for the differential screening were derived from poly(A)$^+$RNA isolated from mycelium of *P. infestans* grown *in vitro*, from *P. infestans*-infected potato leaves, and from non-infected potato leaves. Clones have been selected which hybridized with the interaction, but not with the fungal cDNA probe. As a control, filters were hybridized with the plant cDNA probe. This procedure resulted in selection of differentially hybridizing clones (DHC) containing fungal genes whose expression is induced or significantly increased during pathogenesis (Pieterse *et al.*, 1993b).

Four DHCs were characterized in more detail. Two differentially hybridizing clones were shown to contain genes encoding ubiquitin and calmodulin, respectively (Pieterse *et al.*, 1991 and 1993a). A third *in planta* induced gene, *ipi*B, was shown to belong to a gene family encoding glycine-rich proteins (Pieterse *et al.*, 1994a). Expression of *ipi*B was transient, that is expression was only evident in germinating cysts of *P. infestans* and in infection structures at one day post inoculation. Therefore, it was suggested that *ipi*B could be involved in the development of infection structures (Pieterse *et al.*, 1994b). A fourth clone, DHC-O, appeared to contain two nearly identical *in planta* induced genes, *ipi*O1 and *ipi*O2 (Pieterse *et al.*, 1994a). *Ipi*O genes revealed a transient expression pattern with highest expression at two days post inoculation. The *ipi*O genes reveal no homology with sequences present in data bases. The mature IPI-O proteins, however, contain a tripeptide, Arg-Gly-Asp (RGD), which in mammalian extracellular matrix proteins functions as cell attachment sequence. It has to be proven whether the RGD motif in the IPI-O protein is functional. If this appears to be the case, then the IPI-O proteins may be involved in attachment of *P. infestans* to the plant

surface during the early phases of infection, or interfere with RGD-mediated signalling in plants (Pieterse *et al.*, 1994a; Schindler *et al.*, 1989; Zhu *et al.*, 1993). Gene replacement or anti-sense approaches will have to be employed to determine functions for both *ipi*B and *ipi*O genes during the infection process.

3.2. *In planta* induced genes of the rice pathogen *Magnaporthe grisea*

Several aspects of the interaction between *Magnaporthe grisea* and rice plants have been studied in detail to dissect the infection process. Melanin has been shown to be involved in the penetration of the plant cell wall by a build-up of turgor pressure in the appressorium prior to penetration (Howard and Ferrari, 1989). Penetration of the cuticle by the fungus does not seem to depend on cutin-degrading enzymes as transformation-mediated disruption of the cutinase gene in *M. grisea* did not result in reduced pathogenicity on rice (Sweigard *et al.*, 1992a and 1992b).

A differential screening strategy was developed which was comparable to the method described for *P. infestans* (Talbot *et al.*, 1993). A cDNA library from *M. grisea*-infected rice plants was differentially hybridized with cDNA probes derived from poly(A)$^+$RNA of uninfected rice leaves and of *M. grisea*-infected rice leaves. Several clones were selected containing genes of either fungal or plant origin of which expression was induced upon infection. A cDNA clone pNJT-15 was shown to encode a fungal protein (MPG1) of which the transcript accumulated 60 fold higher during growth of *M. grisea* in rice plants compared to growth *in vitro*. The *MPG1* gene showed a high biphasic expression pattern at 12 h and 72-96 h post inoculation, respectively. Expression of the *MPG1* gene was also observed *in vitro* during nutrient starvation and during conidiation. Inactivation of *MPG1* by gene-disruption resulted in reduced pathogenicity of the *M. grisea* mutants on rice. Detailed morphological analysis of the mutant phenotype revealed reduced ability to undergo infection-related morphogenesis, as well as disturbance in growth during conidiation. The *MPG1* gene encodes a hydrophobin-like protein, a group of secreted fungal proteins which has been described recently (Wessels *et al.*, 1991). For the infection of *M. grisea* on rice, a role for MPG1 has been suggested in attachment, infection court preparation or topological signalling (Talbot *et al.*, 1993). An additional role for MPG1 resulting from the induced expression at 72 h post inoculation, might involve phytotoxic effects, reminiscent of the recently described toxin cerato-ulmin, another hydrophobin (Stringer and Timberlake, 1993). It is clear that further research is needed to establish a working mechanism for MPG1 in appressorium formation and other aspects of the infection process.

4. MOLECULAR CHARACTERIZATION OF THE INTERACTION BETWEEN *CLADOSPORIUM FULVUM* AND TOMATO

The interaction between the pathogenic fungus *Cladosporium fulvum* and tomato has been studied extensively over the last decades (reviewed by Van den Ackerveken and De Wit, 1994b). Infection is initiated as conidia germinate at high humidity on the tomato leaf and the developing runner hyphae grow randomly over the leaf surface (De Wit, 1977). Approximately 3 days after inoculation, fungal hyphae penetrate the tomato leaf through stomata and growth of the fungus continues between mesophyll cells in the intercellular space (De Wit, 1977; Lazarovits and Higgins, 1976a and 1976b). Fungal growth proceeds for several days in the apoplast and hyphae emerge from the leaf, through stomata on the lower side about one week after penetration, to form conidiophores which produce conidia.

The infection cycle of *C. fulvum* is only completed in susceptible but not in resistant tomato plants. In tomato at least eleven genes for resistance against *C. fulvum* have been described. Depending on the resistance gene / avirulence gene combination, fungal growth is arrested at different stages of infection (Hammond-Kosack and Jones, 1994). Resistance is correlated with a hypersensitive response (HR), callose deposition near infection sites (De Wit, 1977; Lazarovits and Higgins, 1976a and 1976b), accumulation of phytoalexins (De Wit and Flach, 1979) and other defence responses.

Growth of *C. fulvum* in susceptible tomato genotypes is strictly confined to the intercellular space of the leaf. No specialized infection or feeding structures are formed and *C. fulvum* retrieves its nutrients directly from the apoplast. The fungus invades the intercellular spaces of tomato leaves without induction of defence responses. Only at later stages of infection, defence responses are induced in compatible interactions as well, most likely caused by stress resulting from occupation of intercellular spaces by the fungus and deregulation of opening and closure of stomata.

The interaction between *C. fulvum* and tomato has been characterized biochemically by analyzing different compounds present in the apoplast of *C. fulvum*-infected tomato leaves. As growth of the fungus is confined to the intercellular space of tomato leaves, apoplastic fluid, obtained after *in vacuo* infiltration and centrifugation of infected leaves, contains various proteins involved in molecular communications between the fungal pathogen and the plant (De Wit *et al.*, 1989). Proteins that accumulate in the apoplast during the infection process in either compatible or incompatible interactions, have been isolated and characterized in detail (Fig. 1) (De Wit *et al.*, 1985 and 1986; De Wit and Spikman, 1982; De Wit and Toma, 1986; De Wit and Van der Meer, 1986; Joosten *et al.*, 1990; Joosten and De Wit, 1988 and 1989; Linthorst *et al.*, 1991; Scholtens-Toma *et al.*, 1989; Scholtens-Toma and De Wit, 1988). Several of these proteins, originating either from the plant or the fungus have been purified, and subsequently, their encoding genes have been cloned. Detailed

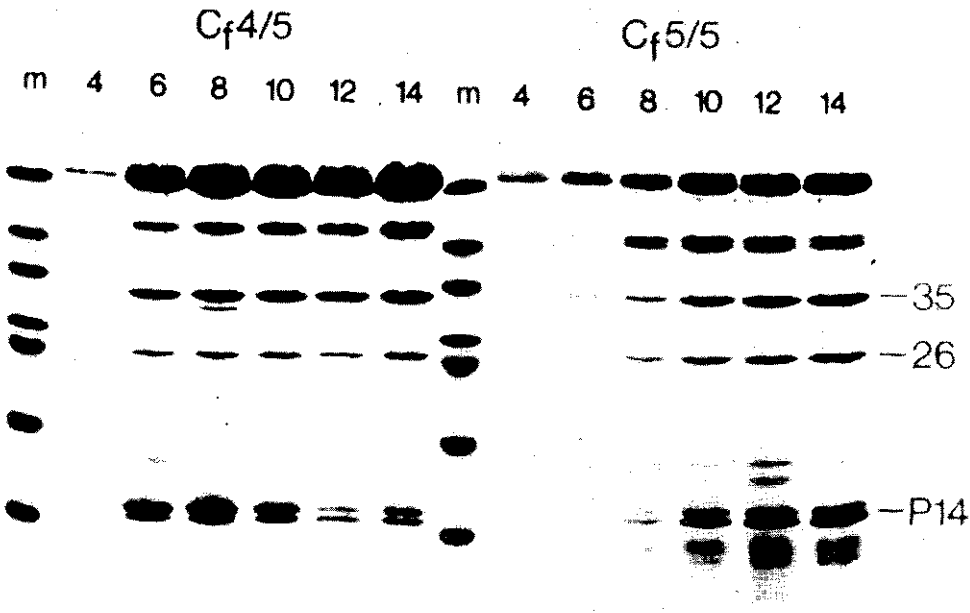

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of apoplastic fluids obtained 4, 6, 8, 10, 12, and 14 d after inoculation of tomato cultivars Cf4 and Cf5 with race 5 of *C. fulvum*. Lanes marked 'm' contain M_r markers (66, 45, 36, 29, 24, 20.1, and 14.2 kD, respectively). Indicated are the 35 kD, extracellular, class II 1,3-ß-glucanase (PR-2a) ('35'), the 26 kD, extracellular, class II chitinase (PR-3a) ('26') and a mixture of the 15 kD, extracellular PR-1a and PR-1b ('P14'). Reprinted with permission from Joosten and De Wit (1989).

analyses of these genes and their products are discussed in the following sections.

4.1. Proteins of *C. fulvum* accumulating in the apoplast during growth *in planta*

Analyses of apoplastic fluid of compatible interactions between *C. fulvum* and tomato revealed accumulation of several small proteins (MW less than 20 kD) in all compatible interactions, irrespective of the races involved (De Wit *et al.*, 1986; Joosten and De Wit, 1988; Wubben *et al.*, 1994). A number of these proteins have been purified and they appeared to be extracellular, fungal proteins named, ECP1, ECP2, and ECP3. Genes encoding ECP1 and ECP2 have been isolated from a cDNA library, made of poly(A)$^+$RNA isolated from *C. fulvum*-infected leaves. For the isolation of ECP1, degenerated primers were used, based on ECP1 amino acid sequences, whereas for the isolation of ECP2, polyclonal antibodies specific for the ECP2 protein were used (Van den Ackerveken *et al.*, 1993). DNA and protein sequence information revealed no homology for *ecp2* with other sequences from data libraries. The *ecp1* gene, however, encodes a protein with a cysteine repeat, reminiscent

of cysteine repeats found in tumour necrosis factor receptor (TNFR) in mammalian systems (Bazan, 1993). TNFRs bind cytokines, which can stimulate a defence response in lymphocytes. Until now, however, it is unknown whether plants contain cytokine-like molecules and whether ECP1 exhibits a receptor-like function. Moreover, TNFRs are usually membrane bound, while ECP1 is a soluble protein secreted by the fungus.

Northern analysis revealed the absence of *ecp*1 gene expression in hyphae of *C. fulvum* grown in liquid shake cultures (*in vitro*), however, during infection *ecp*1 gene expression was strongly induced (Van den Ackerveken *et al.*, 1993). *Ecp*2, is expressed at low level *in vitro*, while a similar *in planta* induced expression has been found as observed for *ecp*1 (Van den Ackerveken *et al.*, 1993). Functions of these proteins in pathogenesis of *C. fulvum* on tomato are not known yet. Gene disruption and/or gene replacement experiments did not result in reduced pathogenicity on young tomato seedlings (Marmeisse *et al.*, 1994; Van den Ackerveken *et al.*, unpubl. results). Possible synergistic action between ECP1 and ECP2 is presently being analyzed, for a *C. fulvum* strain in which both *ecp* genes have been disrupted.

4.2. Specific inducers of host defence responses (race-specific elicitors)

Evidence for the occurrence of race-specific elicitors in apoplastic fluid of *C. fulvum*-infected tomato leaves was first described by De Wit and Spikman (1982). Apoplastic fluid of compatible *C. fulvum*-tomato interactions when injected in tomato genotypes resistant to the fungal race originally used for infection, gave necrosis or chlorosis in the exposed leaf area (De Wit and Toma, 1986; Scholtens-Toma *et al.*, 1989). The elicitors are produced only when the fungus grows *in planta* but not *in vitro*. Until now, two race-specific elicitors, AVR4 and AVR9, have been purified from *C. fulvum*-infected tomato leaves (De Wit *et al.*, 1985; Joosten *et al.*, 1994a; Scholtens-Toma and De Wit, 1988).

The AVR9 elicitor appeared to be a 28-amino-acid-peptide which induced strong necrosis when injected in the intercellular space of leaves of tomato genotype Cf9 (Scholtens-Toma and De Wit, 1988). The *Avr*9 gene was isolated from *C. fulvum* using degenerated oligo-nucleotide probes based on the amino acid sequence of the elicitor (Van den Ackerveken *et al.*, 1992; Van Kan *et al.*, 1991). The *Avr*9 gene appeared to be absent in races of *C. fulvum* which are virulent on Cf9 genotypes of tomato (Van Kan *et al.*, 1991). Molecular genetic proof for having cloned a true avirulence gene from *C. fulvum* was obtained by transformation of a fungal race virulent on Cf9 genotypes with the *Avr*9 gene, which resulted in avirulence on Cf9 genotypes (Van den Ackerveken *et al.*, 1992). Furthermore, transformation-mediated disruption of the *Avr*9 gene in a race of *C. fulvum* avirulent on Cf9 genotypes, made this race virulent on this genotype (Marmeisse *et al.*, 1993). These results indicate that the presence of the *Avr*9 gene is solely responsible for

induction of resistance responses in Cf9 genotypes of tomato, resulting in an incompatible interaction.

Comparable results have been obtained with the race-specific elicitor AVR4, which was isolated following a similar strategy as used for the isolation of the AVR9 race-specific elicitor (Joosten *et al.*, 1994a). Protein sequences of the purified AVR4 elicitor were used to design degenerated primers in order to clone the *Avr*4 gene. Transformation experiments showed that the *Avr*4 gene isolated was responsible for avirulence of *C. fulvum* on Cf4 genotypes of tomato. Similar to the *Avr*9 gene, the *Avr*4 gene is expressed by the fungus only during growth *in planta*. Northern analyses, using RNA obtained from Cf4 genotypes of tomato inoculated with races of *C. fulvum* virulent on Cf4, revealed the presence of an *Avr*4-like transcript in all cases. Further analysis, based on polymerase-chain reaction (PCR), using oligo-nucleotide primers flanking the *Avr*4 coding sequence, showed the presence of *avr*4 alleles in races of *C. fulvum* virulent on Cf4 genotypes of tomato. Detailed sequencing showed that these *avr*4 genes contain one or more mutations in the coding sequence, changing at least one amino acid in the elicitor protein (predominantly a cysteine into a tyrosine). Although all *avr*4 alleles are transcribed during infection, AVR4-like proteins have not been detected in intercellular washing fluid, obtained from tomato leaves infected by races virulent on Cf4 genotypes by using polyclonal antibodies specific for the AVR4 protein. This suggests that the altered amino acid residues influence stability or targeting of the protein (M.A.H.J. Joosten, unpublished results). As a consequence the fungal race becomes virulent on Cf4 genotypes as it lacks the functional elicitor protein.

Expression of the *Avr*4 and *Avr*9 genes has been found to be induced upon growth of *C. fulvum* in susceptible genotypes of tomato (Joosten *et al.*, 1994a; Van den Ackerveken *et al.*, 1994a; Van Kan *et al.*, 1991). This is in agreement with the original observations that race-specific elicitors were only found in apoplastic fluid of *C. fulvum*-infected tomato leaves and not in culture filtrates of *C. fulvum* grown *in vitro*. Detailed expression studies, using the reporter gene *Gus* (ß-glucuronidase) fused to the *Avr*9 gene promoter, showed *Avr*9 promoter activity directly upon penetration of the leaf by the fungus in both resistant and susceptible cultivars (Van den Ackerveken *et al.*, 1994a). However, in resistant cultivars fungal growth is inhibited soon after penetration. In susceptible cultivars strong promoter activity has been observed during growth of the fungus in the intercellular space, especially near the vascular tissue. Furthermore, it was found that nitrogen starvation induced *Avr*9 gene expression *in vitro* (Van den Ackerveken *et al.*, 1994a). Promoter analysis of the *Avr*9 gene revealed the presence of nitrogen responsive elements which are recognized by the positive regulatory proteins NIT2 and AREA from *Neurospora crassa* and *Aspergilus nidulans*, respectively. The function of these elements in *Avr*9 gene expression *in planta* is currently under investigation. For the *Avr*4 gene, no starvation induced expression has been observed. Detailed expression analyses using the *Gus*-reporter gene are currently in progress.

4.3. Defence-related plant proteins accumulating in apoplastic fluid of *C. fulvum*-infected tomato leaves

Several plant proteins have been found to accumulate 2-4 days earlier in incompatible interactions between *C. fulvum* and tomato than in compatible interactions (Fig. 1)(De Wit *et al.*, 1986; De Wit and Van der Meer, 1986). These proteins were identified as pathogenesis-related (PR) proteins (Joosten *et al.*, 1990; Joosten and De Wit, 1989; Linthorst *et al.*, 1991). PR protein accumulation has been observed in many plant species, induced upon infection by pathogenic organisms such as viruses, bacteria and fungi (Linthorst, 1991; Stintzi *et al.*, 1993). The initially identified PR protein were grouped mainly on the basis of their mobility on a native polyacrylamide gel (Linthorst, 1991; Stintzi *et al.*, 1993; Van Loon *et al.*, 1987). More recently, the different groups were subdivided in classes based on gene structure and sequence homology with proteins within the same group or between different groups (Van Loon *et al.*, in preparation). Since PR proteins accumulate in plants upon pathogen infection and are usually associated with acquired resistance (Pan *et al.*, 1991; Pan and Kuć, 1992; Ward *et al.*, 1991), it has been suggested that they are involved in defence of plants against pathogens. In the following sections, PR proteins belonging to different groups will be described briefly. In addition, the accumulation of these proteins in the interaction between *C. fulvum* and tomato, will be discussed (Table 1).

Table 1. Pathogenesis-related (PR) proteins induced in tomato by *Cladosporium fulvum* infection

Family	Member (class)	Molecular weight (kD)	Isoelectric point	Properties	Formerly also known as
PR-1	a	15	10.7	unknown	P4
	b	15	10.9	antifungal	P6, P14
PR-2	a(II)	35	6.4	1,3-ß-glucanase	P3
	b(III)	33	>10		P5
PR-3	a(II)	26	5.0	chitinase	
	b(I)	30	9.0		
	c(I)	32	>10		
	d(II)	27	3.9		
PR-4	a(II)	13	>10	similar to potato *win* proteins	P2
PR-5	a	24	7	thaumatin-like	osmotin

(Adapted from Van Loon *et al.*, in preparation)

4.3.1. PR-1 proteins

PR-1 proteins were first identified in TMV-infected tobacco plants (Gianinazzi *et al.*, 1977; Van Loon, 1976). The function of PR-1 proteins in plant defence against pathogen attack is unclear (Linthorst, 1991; Stintzi *et al.*, 1993). Enzyme activity for PR-1 proteins has not been detected and nucleotide or amino acid sequences do not reveal any information on possible functions.

Two proteins of the PR-1 group were isolated from apoplastic fluid of *C. fulvum*-infected tomato leaves (Fig. 1; Table 1), tomato PR-1a and tomato PR-1b (De Wit and Van der Meer, 1986; Joosten *et al.*, 1990). cDNA clones of the two proteins have been isolated from an expression library, using polyclonal antibodies raised against tomato PR-1b, which also recognise tomato PR-1a (Joosten *et al.*, 1990; Van Kan *et al.*, 1992). Northern analyses showed expression of the genes encoding PR-1a and PR-1b, 2-4 days earlier in incompatible interactions between *C. fulvum* and tomato than in compatible interactions, suggesting a role for PR-1 proteins in the defence of tomato against *C. fulvum* (Van Kan *et al.*, 1992). However, direct *in vitro* antifungal activity for PR-1 proteins against *C. fulvum* has not been observed.

Recently, *in vitro* antifungal activity against *Phytophthora infestans* has been reported for PR-1 proteins (Niderman *et al.*, 1993). In addition, transgenic tobacco plants overexpressing tobacco PR-1a, showed increased resistance against two oomycetous pathogens (Alexander *et al.*, 1993). These observations suggest that PR-1 proteins function in defence of plants against oomycetous pathogens, but no effect of PR-1 proteins has been observed against *C. fulvum*. The mechanism by which PR-1 proteins inhibit growth of Oomycetes is not known. Moreover, a role for PR-1 proteins in active defence of tomato against *C. fulvum in planta,* cannot be excluded.

4.3.2. PR-2 proteins, 1,3-ß-glucanases

PR-2 proteins (1,3-ß-glucanase) have been shown to catalyse the hydrolysis of 1,3-ß-glucan polymers (Kauffmann *et al.*,1987; Kombrink *et al.*, 1988). This indicates the possibility that PR-2 proteins play a role in plant defence, targeted against fungal pathogens with 1,3-ß-glucan containing cell walls. Basic, vacuolar isoforms of 1,3-ß-glucanase (class I) possessed in general higher specific activity than acidic, extracellular isoforms (class II). In addition to the potential antifungal activity, a role for these enzymes in development of healthy plants has been suggested, in view of the expression of PR-2 and PR-2-like genes in flowers and roots (Hennig *et al.*, 1993; Memelink *et al.*, 1990; Ori *et al.*, 1990).

In tomato leaves at least three 1,3-ß-glucanases accumulate after infection by *C. fulvum* (Joosten and De Wit, 1989; Van Kan *et al.*, 1992). Two of these, an apoplastic, acidic, 35 kD 1,3-ß-glucanase (class II)(Fig. 1) and a vacuolar, basic, 33 kD isoform (homologous to the class III extracellular PR-Q' of tobacco [Domingo *et al.*, 1994; Payne

et al., 1990]), have been isolated (Table 1) (Joosten and De Wit, 1989; Van Kan *et al.*, 1992). Two different genes encoding tomato 1,3-ß-glucanases have been cloned, one encoding the acidic, extracellular 35-kD isoform (class II), and one encoding a basic, vacuolar isoform of which the protein has not been identified yet (class I)(Van Kan *et al.*, 1992). Differentially induced gene expression in compatible and incompatible *C. fulvum*-tomato interactions, as observed for PR-1, has been found only for class II, 1,3-ß-glucanase genes (Van Kan *et al.*, 1992) but not for class I, 1,3-ß-glucanase genes. Class I 1,3-ß-glucanase genes were induced to similar extent in compatible and incompatible interactions of *C. fulvum* and tomato (Van Kan *et al.*, 1992). This suggests that only extracellular 1,3-ß-glucanases are involved in active defence of tomato against *C. fulvum*. Although only the vacuolar 1,3-ß-glucanases isolated from *C. fulvum*-infected tomato leaves, exhibit *in vitro* antifungal activity against *Trichoderma viride*, no activity for 1,3-ß-glucanases from tomato was found against *C. fulvum* (Joosten *et al.*, submitted 1994b). Recently, it has been reported that purified 1,3-ß-glucanases from different plant species were able to inhibit growth of several fungi *in vitro* (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993) indicating a possible involvement in active defence of plants against pathogenic fungi. A role for 1,3-ß-glucanases in active defence of tomato against *C. fulvum in planta* remains to be elucidated.

4.3.3. PR-3 proteins, chitinases

PR-3 proteins (chitinases) are able to catalyse the hydrolysis of chitin (Kombrink *et al.*, 1988; Legrand *et al.*, 1987), a structural polymer in cell walls of many fungi (Wessels and Sietsma, 1981). Since chitin does not seem to occur in plants, it has been suggested that chitinases produced by plants upon infection by pathogens play an important role in active defence against pathogenic fungi (Schlumbaum *et al.*, 1986). However, in addition to a potential antifungal activity, the involvement of chitinases in carrot embryogenesis has been demonstrated (De Jong *et al.*, 1991). Furthermore, it has been suggested that the activity of Nod factors, involved in induction of root nodule formation, may partly be determined by the action of chitinases (Staehelin *et al.*, 1994).

Four different chitinases have been purified from *C. fulvum*-infected tomato leaves (Table 1) (Joosten and De Wit, 1989; Joosten *et al.*, submitted 1994b). These consist of two acidic isoforms which occur in the apoplast, and two basic isoforms accumulating intracellularly (Joosten and De Wit, 1989). Genes corresponding to three chitinases have been isolated from tomato (Danhash *et al.*, 1993), one encoding a class I chitinase (PR-3b [chi9]), and the remaining two genes encode class II isoforms (PR-3a [chi3]; PR-3d [chi17]). Northern analyses revealed that only the class II chitinase genes exhibited a differential expression pattern in compatible and incompatible *C. fulvum*-tomato interactions, similar to the pattern observed for the class II 1,3-ß-glucanase genes.

In vitro antifungal activity against *Trichoderma viride* has been observed only for the class I chitinases of tomato. No antifungal activity for any of the chitinases was found against *C. fulvum* (Joosten *et al.*, submitted 1994b). *In vitro* antifungal activity of chitinases of other plant species has been observed against several fungi (Collinge *et al.*, 1993; Mauch *et al.*, 1988; Roberts and Selitrennikoff, 1988; Sela-Buurlage *et al.*, 1993). Inhibition of fungal growth was often correlated with lysis of hyphal tips. The highest *in vitro* antifungal activity has been reported for the vacuolar isoforms of chitinases (class I). Moreover, class I chitinase-overexpressing transgenic plants showed increased resistance against the fungal pathogen *Rhizoctonia solani* (Broglie *et al.*, 1991; Lawton *et al.*, 1993). 1,3-ß-Glucanases and chitinases possessed synergistic antifungal activity in an *in vitro* assay (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). Furthermore, combined overexpression of 1,3-ß-glucanase and chitinase genes in tomato resulted in plants with higher resistance against *Fusarium solani* f.sp. *lycopersici*, than plants overexpressing the chitinase genes only (Van den Elzen *et al.*, 1993).

Although production of chitinases in tomato is highly induced during infection by *C. fulvum*, these proteins do not seem to exhibit direct antifungal activity against this pathogen in an *in vitro* assay. Whether combined induction of chitinase and 1,3-ß-glucanase by an avirulent race of *C. fulvum*, in concert with induction of other defence response-related proteins in tomato, is sufficient to explain successful defence is still unknown.

4.3.4. PR-4 proteins

PR-4 proteins have been isolated from different plant species upon infection by pathogenic organisms (Broekaert *et al.*, 1990; Friedrich *et al.*, 1991; Hejgaard *et al.*, 1992; Joosten *et al.*, 1990; Linthorst *et al.*, 1991; Ponstein *et al.*, 1994). Subdivision of PR-4 proteins in class I and class II, is based on the presence of an N-terminal chitin binding (hevein) domain in the class I PR-4 proteins. Class II PR-4 proteins from tobacco and tomato show homology with the C-terminal domain of the gene products of two wound-inducible genes from potato, *win*1 and *win*2, and with prohevein from *Hevea brasiliensis*, but they lack the hevein domain (Linthorst *et al.*, 1991). Recently, two chitin binding proteins CBP-N and CBP-20 have been purified from barley and tobacco, respectively, and have been characterized as PR-4 proteins as well (Hejgaard *et al.*, 1992; Ponstein *et al.*, 1994).

One PR-4 protein has been isolated (PR-4a) from apoplastic fluid of *C. fulvum*-infected tomato leaves (Joosten *et al.*, 1990) and classified as a class II PR-4 protein (Table 1). Northern analysis revealed increased PR-4a transcript accumulation several days earlier in incompatible interactions between *C. fulvum* and tomato than in compatible interactions. The expression pattern showed much similarity with the pattern observed for PR-1 and the class II PR-2 and PR-3 genes. Unfortunately no antifungal assays have been performed with tomato PR-4a against *C. fulvum*.

The PR-4 proteins CBP-N and CBP-20, were found to possess antifungal activity against *Trichoderma harzianum*, *T. viride* and *F. solani*. Furthermore, synergistic antifungal activity has been found for these PR-4 proteins when applied in combination with class I chitinase or 1,3-ß-glucanase. CBP-20 was shown to contain a hevein domain explaining the chitin affinity (class I, [Ponstein *et al.*, 1994]), in contrast with CBP-N, which irrespectively of its chitin binding affinity, did not contain a hevein domain (class II, [Hejgaard *et al.*, 1992]). Therefore, both class I and class II PR-4 proteins reveal *in vitro* antifungal properties. The mechanism by which PR-4 proteins exhibit antifungal activity is unknown. Furthermore, the role of the tomato PR-4 protein in active defence against *C. fulvum* has not been examined in detail.

4.3.5. PR-5 proteins, thaumatin-like proteins

Proteins of the PR-5 group, initially isolated from the apoplast of TMV-infected tobacco leaves, showed high homology with thaumatin, a sweet tasting protein from the African shrub *Thaumatococcus daniellii* (Cornelissen *et al.*, 1986). In addition, vacuolar proteins produced in several plant species upon osmotic stress by salt (osmotins), showed homology with thaumatin as well (Stintzi *et al.*, 1991). A proposed role for PR-5 proteins in plant defence against fungi has evolved from the homology of PR-5 proteins with permatins. Permatins are proteins isolated from seeds of different plant species which have antifungal activity presumably by permeabilizing the plasma membrane of the fungus (Vigers *et al.*, 1991 and 1992). In agreement with these findings was the observation that AP24 and NP24, two class I PR-5 proteins isolated from tobacco and tomato plants, respectively, showed antifungal activity against *Phytophthora infestans*, correlated with lysis of hyphal tips (Woloshuk *et al.*, 1991). Furthermore, potato plants overexpressing a class I osmotin gene isolated from tobacco showed delayed disease symptom development upon infection by *P. infestans*, but not by *Phytophthora parasitica* var. *nicotianae*, when compared with infected non-transformed potato plants (Liu *et al.*, 1994).

PR-5 proteins have not been identified in *C. fulvum*-infected tomato. Expression studies, however, revealed the induction of osmotin (PR-5) gene expression in tomato upon inoculation with *C. fulvum*. Whether the product of the induced gene is involved in active defence of tomato against *C. fulvum* is unknown.

In summary, the early accumulation of apoplastic PR proteins in incompatible *C. fulvum*-tomato interactions, together with the induced gene expression of the corresponding genes, suggested a role for these PR proteins in the defence response of tomato against *C. fulvum*. Vacuolar, class I, PR-2 and PR-3 protein gene expression has been found to be induced to the same extent in compatible and incompatible interactions. However, *in vitro* growth inhibition assays revealed no antifungal properties for either class I or class II tomato PR-2

and PR-3 proteins, against *C. fulvum*. Only class I, PR-2 and PR-3 proteins from tomato showed *in vitro* antifungal activity against *T. viride*. In combination, these results do not indicate an important role for PR-2 and PR-3 proteins in active defence of tomato against *C. fulvum*. Differences at the cellular level, which could not be established in our biochemical assays, might still result in a positive contribution of PR-2 and PR-3 proteins in the defence response of tomato against *C. fulvum*.

Although, no clear evidence is available on a role for PR proteins in active defence of tomato against *C. fulvum*, results obtained by other research groups indicate that PR proteins can play an important role in plant defence against pathogens. This has been shown for instance by *in vitro* antifungal assays and by PR protein-overexpressing transgenic plants. It is likely that plants respond upon pathogen infection by initiating a complex set of defence responses. Each separate response, such as PR protein production is likely not always effective against the intruding pathogen. The concerted induction of the whole range of defence-related proteins might result in successful defence.

5. CONCLUDING REMARKS

The results obtained by the molecular characterization of the interaction between *C. fulvum* and tomato, have clearly demonstrated the possibilities of a non-biased approach to isolate proteins and their encoding genes, expressed during, and maybe involved in, the infection process. This approach works for other fungal pathogens as well as has been shown for the interactions between *Phytophthora infestans* and potato, and *Magnaporthe grisea* and rice, respectively. The latter research resulted in the cloning and characterization of the pathogenicity gene *MPG1*, by differential hybridization (Talbot *et al.*, 1993). In addition, further developments in the molecular characterization of the *Phytophthora infestans*-potato interaction can be expected in near future, by unravelling the functions of the *in planta* induced genes that were isolated by differential screening (Pieterse *et al.*, 1993b). A prerequisite for non-biased screenings is the availability of molecular genetic techniques, such as a transformation system and the possibility of gene disruption, in order to study in greater detail functions of genes which are isolated. Furthermore, homology of isolated genes with genes present in data bases will facilitate unravelling their functions. Differential screening can result in the isolation of proteins and genes with often unexpected functions and will therefore generate new insights in molecular communication between plant and pathogen.

6. AIM AND OUTLINE OF THIS THESIS

Sustainable agriculture requires various approaches for control of fungal diseases. An important aspect is the development of new disease-resistant cultivars, obtained either by

conventional or molecular breeding for resistance. Basic knowledge on plant-pathogen interactions at the molecular level is a requirement for the development of new strategies for resistance breeding. This would include unravelling the working mechanism of resistance genes, separating the signalling pathways leading to induction of active defence responses, and identifying different aspects of the active defence response. In addition, new leads for molecular breeding for resistance can be obtained by studying fungal pathogenicity at the molecular level. In this thesis we describe the subcellular localization of fungal and plant proteins possibly involved in the interaction between the pathogenic fungus *Cladosporium fulvum* and tomato.

Research on the interaction between *C. fulvum* and tomato, resulted in isolation and characterization of several proteins which accumulate in the intercellular space of *C. fulvum*-infected tomato leaves and which are possibly involved in communication between plant and pathogen. Only for the race-specific elicitors, AVR4 and AVR9, produced by *C. fulvum*, at least one biological function in the infection process is known. However, it is assumed that these proteins, in addition to eliciting an active defence response in resistant tomato genotypes, must have other intrinsic functions for the fungus itself. Furthermore, several proteins which accumulate in the apoplast of *C. fulvum*-infected tomato leaves have to be characterized in more detail to determine their role in pathogenesis, either related with the host-defence response or with fungal pathogenicity. Therefore, we examined the accumulation in time and space of fungal and plant proteins which have been found in the apoplastic fluid of *C. fulvum*-infected tomato leaves.

Chapters 2, 3, and 4 of this thesis deal with immunocytochemical localization of pathogenesis-related proteins which accumulate in tomato leaves upon infection by *C. fulvum*. At the time this research project started, it had been shown that accumulation of apoplastic PR proteins occurred 2-4 days earlier in incompatible interactions than in compatible ones. Some of these PR proteins were shown to have chitinase and 1,3-ß-glucanase activity and were therefore assumed to be potent antifungal proteins. Accumulation of these proteins in time and space in the infected tomato leaf tissue would indicate possible functions specific for the *C. fulvum*-tomato interaction.

The possible degradation of host and fungal cell walls as a result of the accumulation of chitinases and pectolytic enzymes (Aldington and Fry, 1992) in the apoplast of *C. fulvum*-infected tomato leaves has been described in chapter 5.

Detailed analyses of the localized gene expression of class I and class II, chitinases and 1,3-ß-glucanases, in compatible and incompatible interactions between *C. fulvum* and tomato, has been performed using *in situ* hybridization, and has been described in chapter 6. Furthermore, specific induction of PR proteins upon injection of race-specific elicitors, AVR4 and AVR9, in resistant and susceptible tomato leaves, has been analyzed as well.

Chapter 7 describes further characterization of the extracellular fungal proteins, ECP1 and ECP2, which are possibly involved in fungal pathogenicity. *Ecp* gene expression *in planta* has been studied by fusions of the *ecp*-gene-promoters with the *Gus*-reporter gene, and promoter activity has been determined by GUS staining. In addition, immunolocalization of ECP1 and ECP2 in *C. fulvum*-infected tomato leaves has been described as well.

Detailed information on subcellular localization and tissue specific gene expression of the interaction-specific proteins, can give leads towards their functional role during pathogenesis of *C. fulvum* on tomato. Several aspects of plant defence responses with emphasis on new insights on induction of local and systemic resistance have been discussed in the final chapter.

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chapter 2

Accumulation of pathogenesis-related proteins in the epidermis of tomato leaves infected by *Cladosporium fulvum*

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Abstract. Upon infection by *Cladosporium fulvum*, tomato plants start to produce pathogenesis-related (PR) proteins. The PR proteins 1,3-ß-glucanase, chitinase, and PR-1b accumulated near the stomata in the lower epidermis of *C. fulvum*-inoculated tomato leaves as could be determined by immunolocalization with polyclonal antibodies. However, no differences in accumulation of PR proteins between a compatible and an incompatible interaction were found. Results obtained from enzyme activity measurements of 1,3-ß-glucanase and chitinase on similar leaf material as used for the immunolocalization did not fully reflect the immunolocalization data. The antibodies possibly detect only the extracellular but not the intracellular enzymes. The accumulation of PR proteins near the stomata might be part of a general defence response of plants against pathogens and potential pathogens.

Introduction

In their natural environment, plants are challenged by various pathogenic organisms such as bacteria and fungi. Plants are able to defend themselves successfully against most of the potential pathogens and do not show visible disease symptoms. Only a few of the potential pathogenic organisms are able to infect certain plant species.

Upon infection by pathogens, most plants start to produce a group of proteins known as pathogenesis-related proteins (PR proteins). These PR proteins can either be targeted to the vacuoles of the plant cell or secreted to the extracellular space. Over the last decade PR proteins have been studied extensively in order to determine their function in the defence of plants against pathogens (Linthorst, 1991).

Enzymatic functions were found for two groups of PR proteins, namely chitinases and 1,3-ß-glucanases (Kauffmann *et al.*, 1987; Legrand *et al.*, 1987). A role for these hydrolytic enzymes in plant defence against fungal pathogens is suggested as they are able to degrade chitin and 1,3-ß-glucan, major structural elements in the cell walls of many fungi. Therefore, chitinase and 1,3-ß-glucanase are potential inhibitors of fungal growth. Several research groups have found that chitinase and 1,3-ß-glucanase in some cases indeed inhibit growth of fungi *in vitro* (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988). The effect of a few hydrolytic enzymes on fungal growth *in planta* has been studied in transgenic plants which constitutively express the corresponding genes. A reduced sensitivity towards *Rhizoctonia solani* was found in transgenic tobacco expressing a bean chitinase (Broglie *et al.*, 1991). This approach, however, is not effective against all pathogenic fungi since transgenic tobacco expressing high levels of a tobacco chitinase did not show reduced sensitivity towards *Cercospora nicotianae* (Neuhaus *et al.*, 1991a). Thus, the growth of plant pathogenic fungi can not always be inhibited by enhancing the expression of the genes encoding hydrolytic PR proteins in the plant. In order to obtain enhanced pathogen resistance in transgenic plants, correct

targeting of antifungal proteins to the interface between plant and pathogen can be essential (Neuhaus *et al.*, 1991b; Melchers *et al.*, 1993).

Antifungal activity was found for PR proteins other than the hydrolytic PR proteins, e.g. the thaumatin-like proteins of the PR-5 group (Vigers *et al.*, 1991; Woloshuk *et al.*, 1991). Furthermore, it has been shown that transgenic tobacco plants which constitutively produce a tobacco PR-1 protein, show increased resistance against *Peronospora tabacina* (Lawton *et al.*, 1993). The amino acid sequence of the PR-1 proteins does not reveal whether these proteins have enzymatic activity or whether they are structural proteins, and their precise role in the defence of plants against pathogens is unknown.

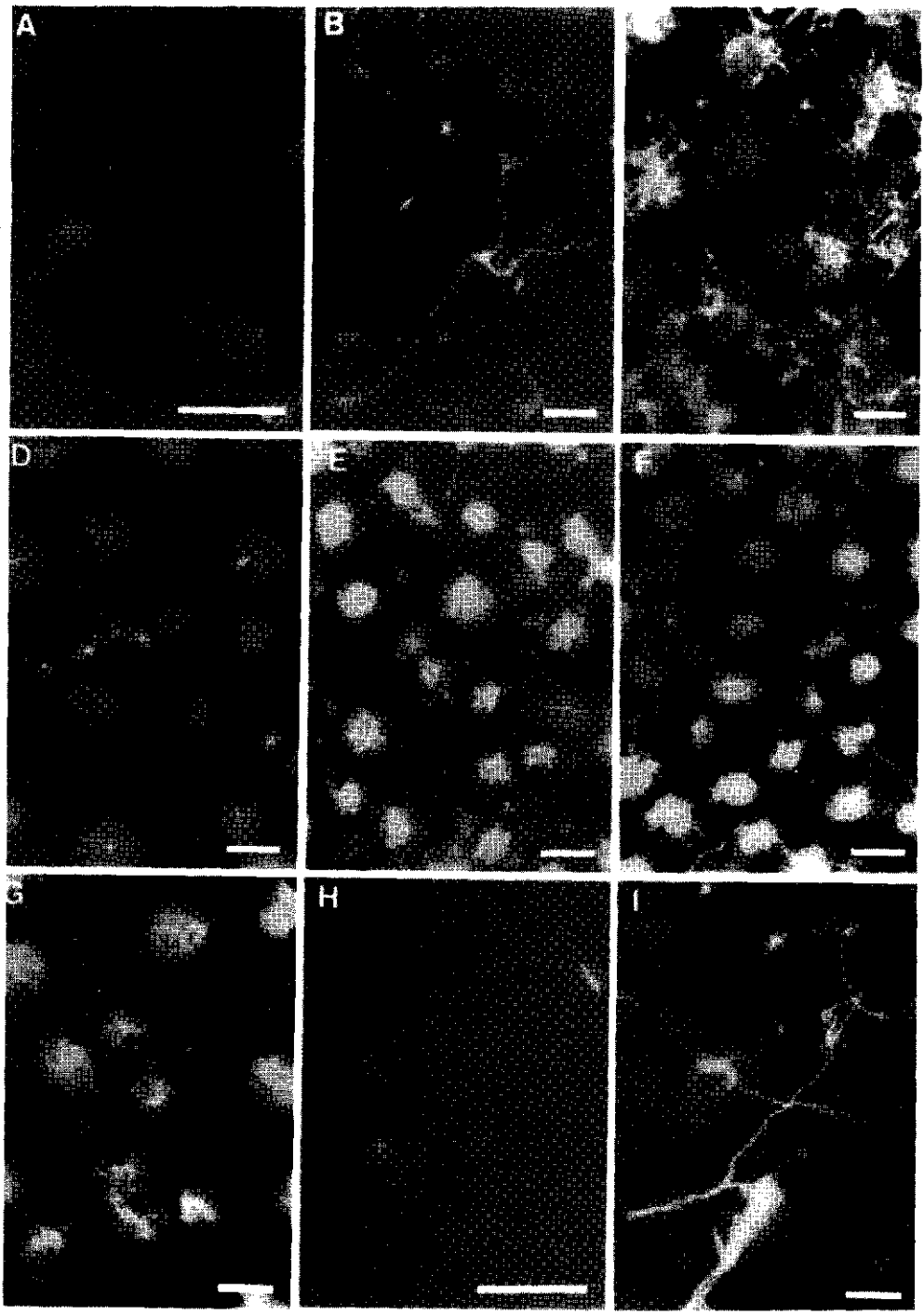
Previously, we studied the distribution of several groups of PR proteins in tomato leaves after inoculation with the fungal pathogen *Cladosporium fulvum*, the causal agent of tomato leaf mould, using electron microscopy. Accumulation of chitinase and 1,3-ß-glucanase has been shown in the vacuoles and in the extracellular space of inoculated tomato leaves (Wubben *et al.*, 1992). Since the fungus enters tomato leaves through stomata preferably at the lower side of the leaf, we were interested in the localization and the accumulation of PR proteins in the lower epidermis of inoculated tomato leaves. In this report we show that a strong accumulation of 1,3-ß-glucanase, chitinase and PR-1b can be observed near the stomata in the lower epidermis of the inoculated leaves. We support these findings with biochemical data and propose a possible function for the accumulation of PR proteins near stomata in the defence of plants against potential pathogens.

Results

Immunolocalization of PR proteins in the lower epidermis

The results of the localization experiments performed on strips of the lower epidermis of inoculated and non-inoculated tomato leaves are shown in Figure 1. In epidermis strips obtained from young healthy plants from the nursery, no accumulation of PR proteins was observed (Fig. 1, A). However, epidermis strips of inoculated leaves revealed a clear accumulation of the PR proteins near the stomata (Fig. 1, C—G). For chitinase (Fig. 1, D) and PR-1b (results not shown), the accumulation could be seen as early as 6 days after

Fig. 1. Immunolocalization of chitinase, 1,3-ß-glucanase and tomato PR-1b on peeled epidermis strips of inoculated and non-inoculated tomato leaves. Immunogold labelling was followed by silverenhancement and the strips were examined with epipolarized light. Silver stain can be observed as bright particles. Localization experiments were performed on non-inoculated leaves (A) and leaves of Cf 5 plants inoculated with race 5 of *Cladosporium fulvum*, leading to a compatible interaction (B, C, D, E, F) and leaves of Cf 4 plants inoculated with race 5 of *C. fulvum* leading to an incompatible interaction (G, H, I). The leaves were examined at 6 (B, D, H) or 12 days (C, E, F, G, I) after inoculation. Labelling was performed with antibodies raised against the 35 kD extracellular 1,3-ß-glucanase (A, B, C), the 26 kD extracellular chitinase (D, E, G), or the 15 kD extracellular PR-1b protein (F). As controls, preimmune serum (H) and antibodies raised against mycelium homogenate of *C. fulvum* (I) were used. Bar = 50 μm.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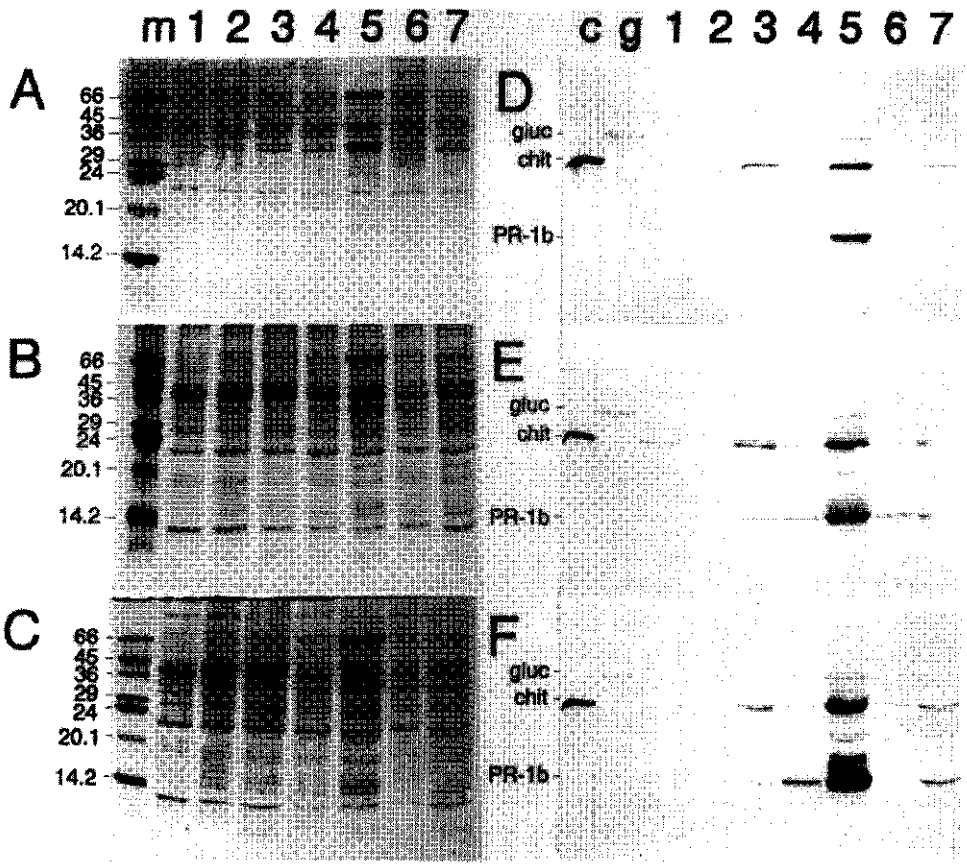

Fig. 2. SDS-polyacrylamide gel electrophoretic profiles (A—C) and western blot analyses of the SDS-polyacrylamide gels (D—F) with preparations obtained from infected and non-infected tomato leaves. The profiles were obtained from homogenates of 10 mg tissue of epidermis strips (A,D), of the remainder of the leaf after removal of the epidermis (B,E), and of total leaf (C,F). The lanes contain molecular weight markers (m), the purified 26 kD chitinase (c), the purified 35 kD 1,3-ß-glucanase (g), preparations of non-inoculated Cf5 plants from the nursery (1), non-inoculated Cf5 plants grown in the same contained environment as the inoculated ones for 6 (2) or 12 days (3), Cf5 plants inoculated with the virulent race 5 at 6 (4) or 12 days (5) after inoculation, and Cf4 plants inoculated with the avirulent race 5 at 6 (6) or 12 days (7) after inoculation. The nitrocellulose membranes were incubated with a mixture of three antibodies which were raised against the purified 26 kD chitinase, the 35 kD 1,3-ß-glucanase and the 15 kD PR-1b protein. The position of these three proteins on the western blots is indicated.

inoculation while the accumulation of 1,3-ß-glucanase was visible only at 12 days after inoculation (Fig. 1, B and C). There was no significant difference in labelling between a compatible and an incompatible interaction. This is only shown for 1,3-ß-glucanase accumulation (Fig. 1, E and G), but similar results were found for chitinase and PR-1b accumulation (results not shown). Accumulation of PR proteins near the stomata could also

be seen in non-inoculated plants which were grown in the same contained environment together with the inoculated plants (results not shown). Control experiments were performed with preimmune serum and with antibodies raised against a homogenate of mycelium of *C. fulvum*. Preimmune serum gave no significant labelling at any stage of infection (Fig. 1, H). With the antibodies raised against homogenate of mycelium of *C. fulvum*, clear labelling of only the fungal hyphae was observed (Fig. 1, I). The antibodies raised against 1,3-ß-glucanase and PR-1b also showed labelling with the fungal hyphae (Fig. 1, B, C and F) suggesting that these plant proteins bind to the fungal hyphae.

Biochemical analyses of PR proteins in leaf tissue

Extracts of the leaves were analysed immunochemically and biochemically in order to be able to distinguish between the different isoforms of 1,3-ß-glucanase and chitinase occurring in tomato plants and to correlate the accumulation of the detected isoforms with the activities measured by the enzyme assays. Homogenates were prepared from 10 mg fresh material of epidermis strips, of the remainder of the leaf after removal of the epidermis, and of the total leaf, and were analysed by SDS-PAGE. By comparing the protein profiles from homogenates of control plants from the nursery (Fig. 2, A—C, lane 1) with those from infected and non-infected plants grown in the same contained environment (Fig. 2, A—C, lanes 2—7), induction of synthesis of several proteins was observed especially in the compatible interaction at 12 days after inoculation (Fig. 2, A—C, lane 5). Furthermore, the accumulation pattern is similar in homogenates from the epidermis of the leaf (Fig. 2, A), the remainder of the leaf after removal of the epidermis (Fig. 2, B) and the total leaf (Fig. 2, C). Western blot analyses with a mixture of antibodies raised against PR-1b, chitinase and 1,3-ß-glucanase, revealed a significant accumulation of these PR proteins in the epidermis of tomato leaves inoculated with *C. fulvum* (Fig. 2, D, lanes 4—7). A similar qualitative pattern of accumulation of PR proteins was observed for the remainder of the leaf after removal of the epidermis (Fig. 2, E, lanes 4—7) and for the total leaf (Fig. 2, F, lanes 4—7). Primarily the 26 kD extracellular chitinase (chit), the 35 kD extracellular 1,3-ß-glucanase (glu) and the 15 kD extracellular PR-1b (PR-1b) are detected on the western blots as indicated. Furthermore, the 27 kD extracellular chitinase, the 30 kD and 32 kD intracellular chitinases, and the 33 kD intracellular 1,3-ß-glucanase were detected and can be observed as 4 distinct bands between the indicated isoforms of chitinase (chit) and 1,3-ß-glucanase (glu) (Fig. 2, E and F, lane 5). A significant amount of PR proteins was detected in non-inoculated plants which were grown in the same contained environment together with the inoculated ones (Fig. 2, D—F, lanes 2 and 3).

Enzyme assays revealed that the activity of hydrolytic enzymes was on average twofold lower in the epidermis than in the remainder of the leaf after removal of the epidermis. The activity of chitinase and 1,3-ß-glucanase was in most cases the highest in the

incompatible interaction at 12 days after inoculation (results not shown). This seems contradictory with the results obtained with western blots where the highest concentration of PR proteins was observed in the compatible interaction at 12 days after inoculation (Fig. 2, D—F, lanes 5), and indicates that the quantity of PR proteins as detected with the antibodies does not fully reflect the enzyme activity as measured in the biochemical assays.

Discussion

Strong accumulation of several PR proteins was observed near the stomata in the lower epidermis of *C. fulvum*-inoculated tomato leaves. The local accumulation of PR proteins in the epidermis of plants which were treated with biotic and abiotic agents has been reported before by several groups (Ohashi and Matsuoka, 1987; Mauch *et al.*, 1992; Kombrink *et al.*, 1993). In an extensive electron microscopic study on the accumulation of chitinases and 1,3-ß-glucanases in ethylene-treated bean leaves, Mauch and coworkers (1992) found significant accumulation of vacuolar chitinases and 1,3-ß-glucanases in the cells of the lower epidermis and in cells adjacent to the vascular tissue. This accumulation was strictly correlated with the formation of protein aggregates in the vacuoles of these cells. Furthermore, the extracellular PR proteins were located in the extracellular space near the stomata and the mesophyll cells, associated with extracellular material. With the antibodies that we have used in this study, the vacuolar and the extracellular isoforms of the chitinases and 1,3-ß-glucanases can not be distinguished (Joosten and De Wit, 1989; Joosten *et al.*, 1990). In previous studies, where we used electron microscopy and immunogold staining, we observed accumulation of chitinases and 1,3-ß-glucanases in protein aggregates in the vacuoles and in extracellular material around mesophyll cells (Wubben *et al.*, 1992). In this study we found accumulation of PR proteins near the stomata in the lower epidermis of the inoculated tomato leaves. Since the localization experiments were performed on intact layers of tissue, it is likely that predominantly extracellular PR proteins were detected as the cytoplasm of intact cells is not accessible to antibodies and gold particles. Furthermore, no difference in accumulation was observed between a compatible and an incompatible interaction. The accumulation of PR proteins near the stomata was also observed in non-inoculated plants which were grown in the same contained environment together with the inoculated plants. Leaves obtained from tomato plants from the nursery, did not accumulate PR proteins. Ethylene or other volatile signal molecules produced by the inoculated plants, might be responsible for triggering responses leading to the accumulation of PR proteins in leaves of non-inoculated tomato plants (Farmer and Ryan, 1990; Mauch *et al.*, 1992).

Western blot analyses and chitinase and 1,3-ß-glucanase activity assays were performed in order to be able to distinguish between the different isoforms of PR proteins which accumulated in tomato leaves (Joosten and De Wit, 1989) and to compare the

localization results with the enzyme activities in the different leaf preparations. Western blots revealed no qualitative difference in accumulation when the results from the lower epidermis were compared with the results from the remainder of the leaf after removal of the epidermis. The amount of accumulated PR proteins was highest in the compatible interaction at 12 days after inoculation. With the enzyme assays, however, in most cases the highest activity was found for the incompatible interaction at 12 days after inoculation. This indicates that proteins that are either of low abundance or poorly detected by the antibodies used, contribute significantly to the overall enzyme activities. The differences in specific activities between the intracellular and extracellular isoforms of chitinases and 1,3-ß-glucanases as found in tobacco might also support this finding (Kauffmann *et al.*, 1987; Legrand *et al.*, 1987; Sela-Buurlage *et al.*, 1993). The antibodies used, were raised against extracellular isoforms of the PR proteins, whereas it is reported that the intracellular isoforms have the highest specific activity.

In the *C. fulvum*-tomato interaction the accumulation of PR proteins in the epidermis does not seem to play a crucial role in determining the final outcome of the interaction since no differences in accumulation were observed between a compatible and an incompatible interaction. However, there might be a role for PR protein accumulation near stomata in defence of plants against potential pathogens. A potential pathogen is confronted with hydrolytic enzymes as soon as it enters the leaf through the stomata. The speed of accumulation and the specific activity of the hydrolytic enzymes can be important factors for a plant to successfully defend itself against potential pathogens.

Materials and methods

Fungus, plants and inoculation

Race 5 of *Cladosporium fulvum* (Cooke) (syn *Fulvia fulva* [Cooke] Cif) was subcultured on potato dextrose agar as described before (De Wit, 1977; De Wit and Flach, 1979) and a suspension of $5 \cdot 10^6$ conidia ml^{-1} was used to inoculate near isogenic lines Cf5 (susceptible to race 5) and Cf4 (resistant to race 5) of tomato (*Lycopersicon esculentum* Mill.). Plants were cultured under greenhouse conditions as described before (De Wit 1977; De Wit and Flach, 1979).

Tissue processing for immunolocalization

Epidermis strips were removed from the lower side of tomato leaves with a curved tip tweezers. The strips were immediately transferred to a freshly prepared solution of 0.7% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.1 M PIPES buffer (pH 7.3) with 3 mM $CaCl_2$. Fixation was performed at room temperature for 2 h. After rinsing the material in 0.1 M PIPES buffer (pH 7.3), the tissue was used for immunolocalization experiments.

Antibodies

Polyclonal antibodies raised against the 26 kD extracellular chitinase (tomato PR-3a), the 35 kD extracellular 1,3-ß-glucanase (tomato PR-2a) and the 15 kD extracellular P6 protein (tomato PR-1b) were prepared as described by Joosten and De Wit (1989) and Joosten *et al.* (1990).

Immunogold labelling

After fixation, the epidermis strips were preincubated for 30 min in a solution of phosphate-buffered saline (pH 7.2) (PBS) containing 1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20 (PBS/BSA/Tween) to prevent non-specific binding of the antibodies. This was followed by incubation for 2 h with the specific polyclonal antibodies, diluted 1:1000 in PBS/BSA/Tween. After washing the epidermis strips twice with PBS, they were incubated for 1.5 h in colloidal gold-conjugated protein A (pAg) with gold particle size of 15 nm (Van Lent and Verduin, 1986) in PBS/BSA/Tween (A_{520nm} = 0.1). Subsequently, the epidermis strips were washed twice with PBS and fixed with 1.0% (v/v) glutaraldehyde in PBS (10 min), followed by rinsing with H_2O. The labelling signal was enhanced with an AURION R-Gent silver enhancement kit (AURION, Wageningen, the Netherlands). Tissue was examined with epi-illumination on a Zeiss Axioscope microscope equipped with an IGS filter block (epipolarization microscopy) and an MC100 camera unit (Carl Zeiss, Germany).

Preparation of leaf homogenate

Leaf material was homogenized with a pestle in a 1.5 ml reaction vial containing 0.5 ml 250 mM NaAc buffer (pH 5.2). The homogenate was centrifuged at 14000 **g** (10 min) at 4 °C. Subsequently, proteins in the supernatant were precipitated in 60% (v/v) acetone at -20 °C for 16 h. The precipitate was pelleted by centrifuging at 1000 **g** (10 min) at 4 °C and after drying redissolved in H_2O.

Electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Joosten and De Wit (1988). For western blots, nitrocellulose membranes (0.2 μm, Schleicher & Schuell) were used and blotting was performed as described by De Wit *et al.* (1986). For immunological detection, goat-anti-rabbit alkaline phosphatase (Pierce) was used with nitro blue tetrazolium chloride (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Sigma) as substrates.

Chitinase and 1,3-ß-glucanase activity assays

Assays for chitinase and 1,3-ß-glucanase activity were performed as described by Joosten and De Wit (1989).

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chapter 3

Subcellular localization of plant chitinases and 1,3-ß-glucanases in *Cladosporium fulvum* (syn. *Fulvia fulva*)-infected tomato leaves

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Physiological and Molecular Plant Pathology **41** (1992) 23-32

Abstract. The pathogenesis-related (PR) proteins, chitinases and 1,3-ß-glucanases, are thought to play an important role in the active defence of plants against various fungal pathogens. Previously we found differential accumulation of these enzymes in compatible and incompatible *Cladosporium fulvum*-tomato interactions. Here we report the localization of chitinase and 1,3-ß-glucanase *in planta* with immunogold labelling techniques. The antibodies which were used, recognize both the intracellular and the extracellular isoforms of the chitinases and the 1,3-ß-glucanases. In compatible and incompatible interactions neither the host cells around the infection sites nor the intact hyphae showed a differential accumulation of chitinases and 1,3-ß-glucanases. However, in incompatible interactions degenerated material possibly of fungal origin, was observed, which showed strong labelling with chitinase and 1,3-ß-glucanase antibodies. Strong intracellular labelling was also found near protein aggregates in the plant vacuoles. Furthermore, electron-dense structures of plant origin which showed very strong labelling with both chitinase and 1,3-ß-glucanase antibodies, were found primarily in the extracellular space in incompatible interactions.

Introduction

Plants are able to defend themselves against fungal pathogens by a range of defence mechanisms among which the production of pathogenesis-related (PR) proteins is one of the most intensively studied (Bol *et al.*, 1990; Boller, 1987; Graham and Graham, 1991; Linthorst, 1991; Van Loon, 1989). PR proteins are known to have intra- and extracellular isoforms with different isoelectric points (Linthorst, 1991). It was first shown for tobacco that some of these PR proteins have chitinase or 1,3-ß-glucanase activity (Kauffmann *et al.*, 1987; Legrand *et al.*, 1987). Since many fungi contain chitin and 1,3-ß-glucans as major structural components of their cell walls (Wessels and Sietsma, 1981), it was suggested that the accumulation of chitinases and 1,3-ß-glucanases plays an important role in plant defence (Boller, 1987). This was demonstrated by *in vitro* growth inhibition of some imperfect fungi and Ascomycetes, by chitinases and 1,3-ß-glucanases isolated from plants (Mauch *et al.*, 1988; Schlumbaum *et al.*, 1986). Furthermore, glucan fractions, produced after the breakdown of fungal cell walls by 1,3-ß-glucanases, have been shown to act as elicitors of defence responses of plants (Keen and Yoshikawa, 1983). As the experiments with 1,3-ß-glucanases and chitinases mentioned above were performed *in vitro*, experimental data are needed to demonstrate the actual accumulation and localization of chitinases and 1,3-ß-glucanases *in planta* during the active defence of plants against fungi. This becomes especially urgent, as presently many research-groups are transforming plants with genes coding for these enzymes with the aim of obtaining fungus-resistant plants (Broglie *et al.*, 1991; Neuhaus *et al.*, 1991).

Studies on the subcellular localization of chitinases and 1,3-ß-glucanases *in planta* can provide important information about the role of the accumulated hydrolytic enzymes during active defence (Benhamou *et al.*, 1989 and 1990; Graham and Graham, 1991; Keefe *et al.*, 1990; Kombrink *et al.*, 1988; Mauch and Staehelin, 1989; Schröder *et al.*, 1992; Sock *et al.*, 1990; Spanu *et al.*, 1989). Sock *et al* (1990) found 1,3-ß-glucanases in wheat (*Triticum aestivum*), infected by wheat stem rust (*Puccinia graminis* f.sp. *tritici*), associated with the outer-layer of host and fungal cell walls. In *Fusarium oxysporum* f.sp.*radicis-lycopersici*-infected tomato roots, a difference in the timing of accumulation of chitinase and 1,3-ß-glucanase at the infection sites was observed between resistant and susceptible cultivars (Benhamou *et al.* 1989 and 1990). Chitinase was found in areas where host walls were in close contact with fungal hyphae. The association of chitinase with fungal cell walls was primarily found at sites where the fungal cell walls were distorted, suggesting that other hydrolytic enzymes or factors affect fungal hyphae before the chitin layer is exposed to chitinases. The fungal chitin is probably embedded in polymers such as proteins, polysaccharides or glycoproteins and is therefore inaccessible to plant chitinases (Benhamou *et al.*, 1990; Spanu *et al.*, 1989).

In tomato (*Lycopersicon esculentum* Mill.) the PR proteins that accumulate in the extracellular space after infection by the fungal pathogen *Cladosporium fulvum* have been studied extensively (De Wit *et al.*, 1986; Joosten and De Wit, 1989; Joosten *et al.*, 1990; Van Kan *et al.*, 1992). As this biotrophic fungus remains confined to the apoplast during the main part of its life cycle (De Wit, 1977; Lazarovits and Higgins, 1976a and 1976b), characterization of the various proteins present in the extracellular space provides information about fundamental aspects of communication between the fungus and its host. Joosten and De Wit (1989) showed the appearance of at least two 1,3-ß-glucanases, a basic one, occurring intracellularly and an acidic one, occurring extracellularly, and four chitinases, of which two basic isoforms occur intracellularly and two acidic isoforms occur extracellularly. There is a clear difference between compatible and incompatible *C. fulvum*-tomato interactions with respect to the accumulation of the extracellular 1,3-ß-glucanase and chitinases (Joosten and De Wit, 1989). The extracellular PR proteins accumulate two to four days earlier in incompatible interactions, suggesting they play a role in the successful defence of the plant. However, studies on the proteins accumulating in the apoplast have not provided information about their actual concentrations at locations around infection sites. Here we report on the localization of chitinases and 1,3-ß-glucanases in compatible and incompatible interactions between *C. fulvum* and tomato. The possible role of both enzymes in the active defence of tomato against *C. fulvum* is discussed.

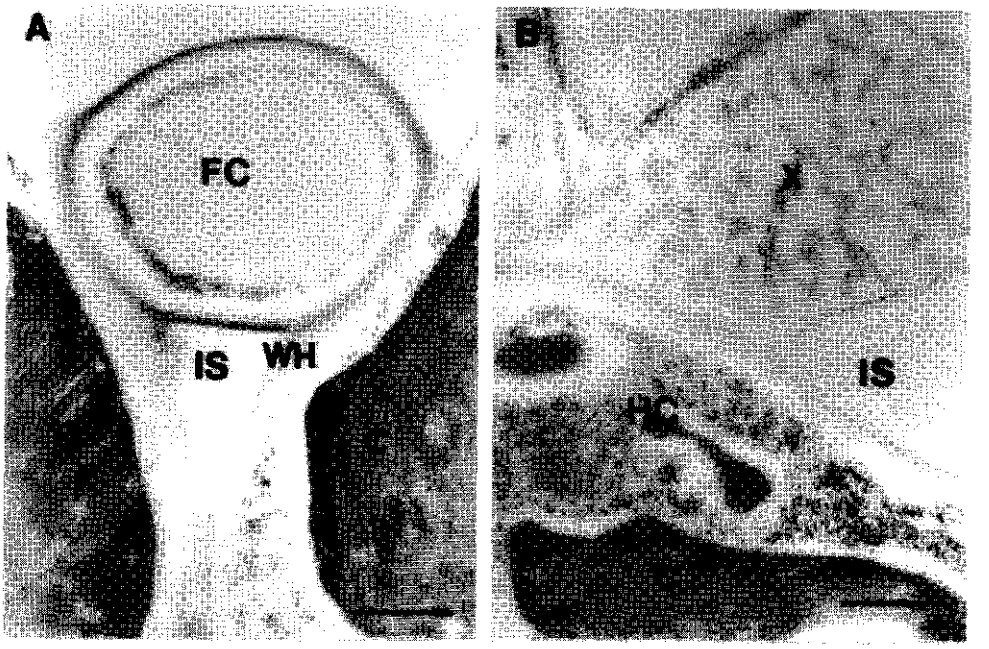

Fig. 1. Immunocytochemical control tests on thin sections of tomato leaf tissue infected by *C. fulvum*. Tomato leaf tissue of (A) a compatible interaction (Cf5/race 5) and (B) an incompatible interaction (Cf4/race 5), obtained 12 days after inoculation, was incubated with preimmune serum. No gold deposition was found in leaf tissue of control tests, neither near the fungal hyphae (FC), the intercellular space (IS), the host cell wall (WH), the cytoplasm of the mesophyll cells (HC), the chloroplasts (C), nor in extracellular material (X) in the apoplast. Bar = 0.5 μm.

Results

Immunocytochemical controls

Figure 1 (A and B) shows that the immunocytochemical controls treated with preimmune serum gave no labelling. Incubation with chitinase or 1,3-ß-glucanase antibodies, preincubated with the corresponding antigen, showed a strong decrease of the labelling intensity indicating that the labelling with the antisera was specific (not illustrated). Incubation with the protein A-gold complex only, also gave no labelling.

Immunogold localization of chitinase and 1,3-ß-glucanase in healthy tomato leaf tissue

Labelling of leaves of non-inoculated tomato plants did not result in significant labelling either with chitinase or with 1,3-ß-glucanase antibodies (not illustrated).

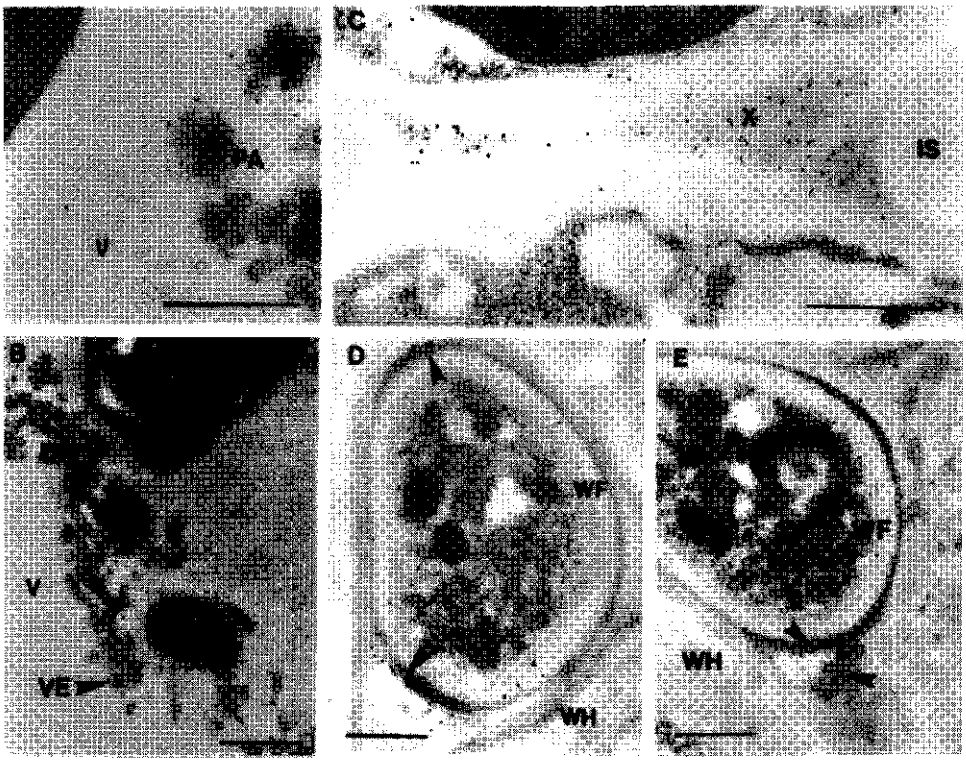

Fig. 2. Immunocytochemical localization of chitinases and 1,3-ß-glucanases in thin sections of susceptible tomato leaf tissue infected by *C. fulvum*. Sections were incubated with antibodies raised against the acidic 26 kD chitinase (B,D) or the acidic 35 kD 1,3-ß-glucanase (A,C,E), followed by incubation with protein A-gold (7 nm). (A) Tomato leaf tissue of a compatible interaction (Cf5/race 5), obtained 12 days after inoculation. A clear deposition of gold particles is observed near the protein aggregates (PA) in the vacuoles (V) of the mesophyll cells. (B) Tomato leaf tissue of a compatible interaction (Cf5/race 5), obtained 5 days after inoculation. Deposition of gold particles in a small vesicle (VE) in the cytoplasm is shown. No gold particles are present in the vacuoles (V) of the host cells or at the endoplasmic reticulum (ER) in the cytoplasm. (C) Tomato leaf tissue of a compatible interaction (Cf5/race 5), obtained 12 days after inoculation. Deposition of gold particles is associated with extracellular material (X) present between two host cells. No labelling was found in the intercellular space (IS) or in the chloroplasts (C) of the mesophyll cells. (D,E) Tomato leaf tissue of a compatible interaction (Cf5/race 5), obtained 10 days after inoculation. In both cases deposition of gold particles is visible at the matrix in the intercellular space (arrows) closely attached to the wall of the fungal hypha (WF). There is no labelling with gold particles at the host cell wall (WH) and only a few gold particles are deposited at the fungal cell wall (WF) (E). Few gold particles are found in the cytoplasm of the host cells adjacent to fungal hyphae. Bar = 0.5 μm.

Immunogold localization of chitinase and 1,3-ß-glucanase in compatible Cladosporium fulvum*-tomato interactions*

The histological characteristics of the interaction between tomato and *Cladosporium fulvum* have already been published by different researchers (De Wit, 1977; Lazarovits and Higgins,

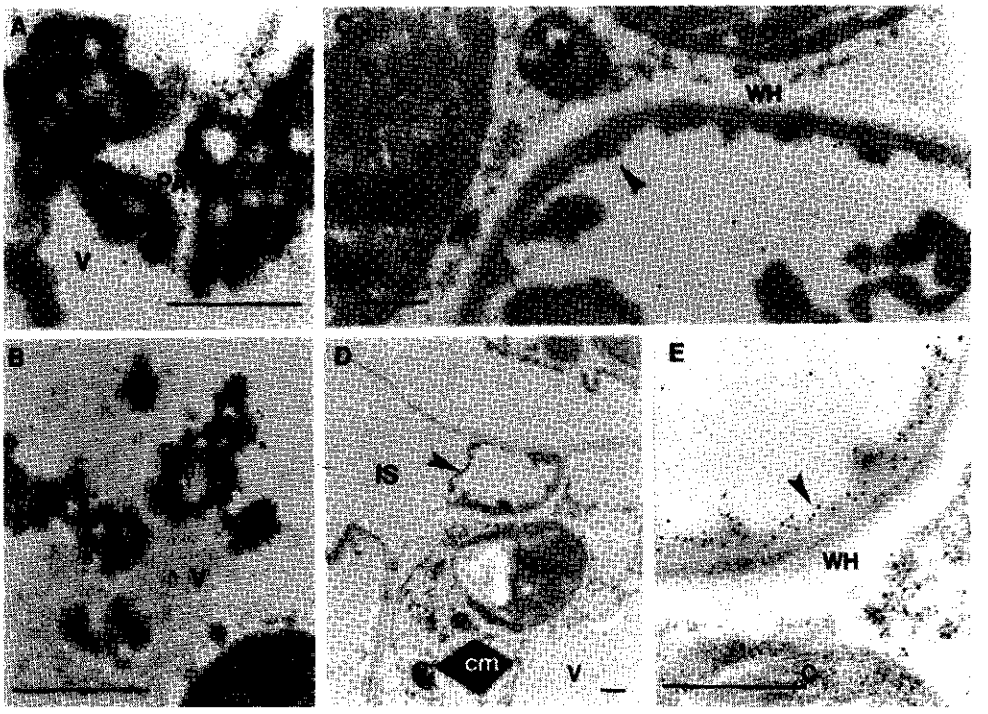

Fig. 3. Immunocytochemical localization of chitinases and 1,3-ß-glucanases in thin sections of resistant tomato leaf tissue infected by *C. fulvum*. Sections were incubated with antibodies raised against the acidic 26 kD chitinase (A,D,E) or the acidic 35 kD 1,3-ß-glucanase (B,C) followed by incubation with protein A-gold (7 nm). (A) Tomato leaf tissue of an incompatible interaction (Cf4/race 5), obtained 12 days after inoculation. Strong deposition of gold particles occurred in protein aggregates (PA) in the vacuoles (V) of mesophyll cells. (B) Tomato leaf tissue of an incompatible interaction (Cf9/race 5) obtained 5 days after inoculation. Strong deposition of gold particles is visible in the protein aggregates (PA) in the vacuoles (V) whereas no labelling was observed in the chloroplast (C). (C,D,E) Tomato leaf tissue of an incompatible interaction (Cf4/race 5) obtained 12 days after inoculation. Electron-dense material in the apoplast (arrows) was found in the incompatible interaction in the intercellular space (IS) at a later stage of infection. A strong deposition of gold particles at this extracellular material was found with both the 1,3-ß-glucanase (C) and the chitinase (D,E) antibodies. There was absolutely no labelling in the wall of the host cell (WH) adjacent to this extracellular material. Furthermore no gold particles were found in the crystalline microbody (cm), the chloroplast (C), the mitochondrion (M) or the host vacuole (V). Bar = 0.5 μm.

1976a and 1976b). In compatible interactions chitinases and 1,3-ß-glucanases were found to accumulate in both intracellular as well as extracellular parts of the tomato leaf (Fig. 2). Intracellularly, labelling was found primarily in electron-dense protein aggregates in vacuoles of the mesophyll cells (Fig. 2, A) but also evenly spread over the cytoplasm (not illustrated) and sometimes even located in small vesicles in the cytoplasm (Fig. 2, B). Chitinases and 1,3-ß-glucanases accumulated in the extracellular space throughout the tissue. Labelling was

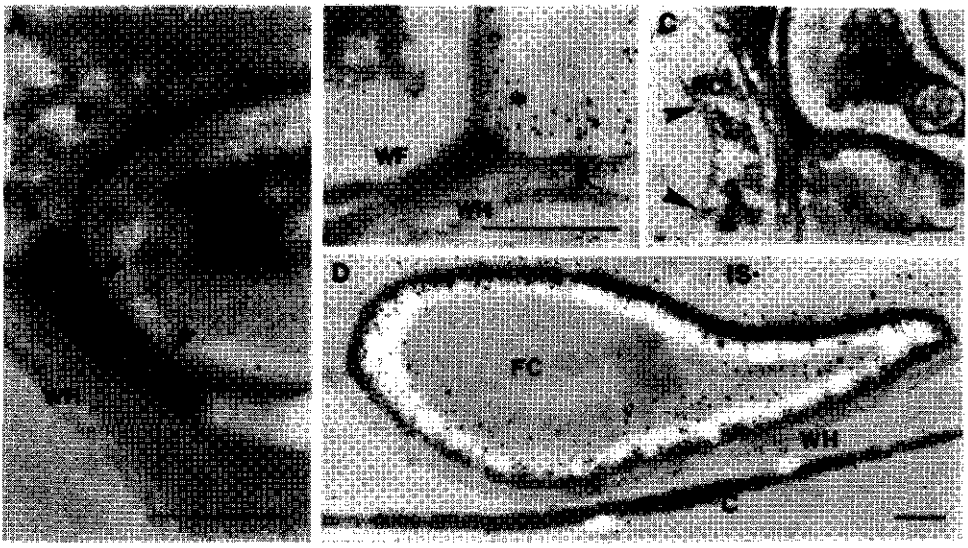

Fig. 4. Immunocytochemical localization of chitinases and 1,3-ß-glucanases in thin sections of resistant tomato leaf tissue infected by *C. fulvum*. Sections were incubated with antibodies raised against the acidic 26 kD chitinase (A,D) or the acidic 35 kD 1,3-ß-glucanase (B,C) followed by incubation with protein A-gold (7 nm). (A,B,C) Tomato leaf tissue of an incompatible interaction (Cf4/race 5) obtained 12 days after inoculation. (A,B) Deposition of gold particles is shown (arrows) near fungal mycelium (FC), especially at the matrix (X) in the intercellular space. No accumulation of gold particles occurred at either the host cell wall (WH) or the cell wall of the fungus (WF). (C) Deposition of gold particles is visible (arrows) in the cytoplasm of the host cell (HC) adjacent to the fungal hypha (FC) and at the matrix (X) in the intercellular space. (D) Tomato leaf tissue of an incompatible interaction obtained 10 days after inoculation. Strong deposition of gold particles was found in an structure, most likely of fungal origin (FM), in the intercellular space (IS). No labelling was found at the host cell wall (WH). Bar = 0.5 μm.

very often associated with an extracellular matrix (Fig. 2, C). Accumulation of chitinases and 1,3-ß-glucanases near fungal hyphae was almost exclusively observed when the fungal cells were tightly surrounded by mesophyll cells (Fig. 2, D and E). In those cases the enzymes seemed to be trapped in the small extracellular spaces surrounding the hyphae.

Immunogold localization of chitinase and 1,3-ß-glucanase in incompatible Cladosporium fulvum*-tomato interactions*

In incompatible interactions, chitinases and 1,3-ß-glucanases also accumulated strongly in protein aggregates in the vacuoles of the mesophyll cells (Fig. 3, A and B). The number of protein aggregates, as well as the labelling intensity found in these aggregates, seemed to be higher in the incompatible interactions compared to the compatible ones. In incompatible interactions we also observed accumulation of chitinase and 1,3-ß-glucanase in the mesophyll cells, surrounding the fungal hyphae (Fig. 4, C). Furthermore, in the incompatible interaction

we observed electron-dense material in the extracellular space which was strongly labelled with both chitinase and 1,3-ß-glucanase antibodies (Fig. 3, C, D and E). The accumulation of chitinases and 1,3-ß-glucanases near apparently intact mycelium in incompatible interactions was comparable with the accumulation in compatible interactions (Fig. 4, A and B). Chitinases and 1,3-ß-glucanases accumulated around the fungal hyphae but no labelling was observed at the fungal cell wall. However, in a few cases, we observed fungal-like structures in incompatible interactions, which gave a very strong labelling with chitinase (Fig. 4, D) and 1,3-ß-glucanase antibodies. Such structures can be partly degraded empty hyphae.

Discussion

The sites where intracellular chitinases and 1,3-ß-glucanases accumulate in mesophyll cells of tomato leaves infected by *C. fulvum* were not significantly different in compatible and incompatible interactions. Chitinase and 1,3-ß-glucanase accumulated in the cytoplasm of mesophyll cells adjacent to fungal hyphae in both compatible and incompatible interactions. Protein aggregates containing significant amounts of chitinase and 1,3-ß-glucanase occurred in the vacuoles of the mesophyll cells. However, in incompatible interactions the protein aggregates occurred in a higher number of cells and they contained a larger amount of chitinase and 1,3-ß-glucanase, compared to compatible interactions. The occurrence of protein aggregates in vacuoles has been reported in various plant tissues following treatment with ethylene or inoculation with mycorrhizal fungi, viruses and viroids (Dore *et al.*, 1991; Keefe *et al.*, 1990; Mauch and Staehelin, 1989; Spanu *et al.*, 1989; Vera *et al.*, 1989). Although the antibodies that we used do not discriminate between acidic and basic 1,3-ß-glucanases and chitinases, the 1,3-ß-glucanases and chitinases associated with the vacuolar protein aggregates are most likely basic, as reported by a number of researchers (Dore *et al.*, 1991; Joosten and De Wit, 1989; Keefe *et al.*, 1990; Linthorst, 1991).

It has been reported before that it is difficult to fix water soluble proteins in the extracellular space with conventional fixation methods (Dore *et al.*, 1991; Sock *et al.*, 1990). Therefore, we have the impression that the labelling observed in extracellular structures as shown in Figure 2 (C) is probably not due to a specific accumulation of chitinases and 1,3-ß-glucanases but rather because these structures can serve as a carrier and immobilize proteins that accumulate in the apoplast.

We observed electron-dense material in the extracellular space which was strongly labelled with both chitinase and 1,3-ß-glucanase antibodies in incompatible interactions. Similar structures were found in virus-infected tobacco in which the occurrence of these extracellular pockets was correlated with the hypersensitive response of tobacco plants (Dore *et al.*, 1991). This electron-dense material found in the apoplast, showed a structural

similarity with protein aggregates present in the vacuoles. Furthermore, at 6 to 8 days after inoculation in incompatible interactions the intracellular basic 1,3-ß-glucanase is detected in apoplastic fluid as shown by denaturing polyacrylamide gel electrophoresis (Joosten and De Wit, 1989). However, with the antibodies we used, it was not possible to discriminate between the acidic and the basic isoforms of chitinases and 1,3-ß-glucanases, which makes it difficult to correlate extracellular pockets with the protein aggregates present in the vacuoles. Dore and coworkers (Dore *et al.*, 1991) showed that the extracellular pockets in tobacco contained mainly the acidic isoforms of the chitinases and 1,3-ß-glucanases suggesting that there is no correlation with the protein aggregates from the vacuoles.

We did not find differential accumulation of apoplastic chitinases or 1,3-ß-glucanases near fungal hyphae in incompatible interactions, when compared with compatible interactions. In both cases, accumulation of these hydrolytic enzymes was observed near intact fungal hyphae, but almost no labelling was found at the fungal cell wall. It has been reported before that fungal chitin and 1,3-ß-glucan are embedded in a matrix of proteins, polysaccharides and glycoproteins which may function as a protective mucilage layer against hydrolytic enzymes (Benhamou *et al.*, 1990; Mauch *et al.*, 1988; Spanu *et al.*, 1989). Hyphal tips, however, were found to be susceptible to chitinases and 1,3-ß-glucanases (Collinge *et al.*, 1993; Mauch *et al.*, 1988). In our observations, fungal hyphae which showed no accumulation of chitinases and 1,3-ß-glucanases at their cell walls, were visibly undamaged. However, in incompatible interactions we occasionally observed fungal-like structures (Fig. 4, D) which were strongly labelled with both chitinase and 1,3-ß-glucanase antibodies. Such structures possibly represent degraded apices of hyphae. It has been reported before that the specific enzymatic activity of both basic chitinases and basic 1,3-ß-glucanases is much higher than that of the acidic isoforms (Kauffmann *et al.*, 1987; Legrand *et al.*, 1987). If the basic vacuolar isoforms of chitinases and 1,3-ß-glucanases are released during the hypersensitive response near the apex of a growing hypha in the incompatible interaction only, they could play an important role in the active defence of tomato plants against *C. fulvum*.

We have the impression that chitinases and 1,3-ß-glucanases only partly determine the outcome of the interaction between tomato and *Cladosporium fulvum*. We and others have reported that phytoalexins and callose accumulate to a higher extent in incompatible than in compatible *Cladosporium fulvum*-tomato interactions (De Wit and Flach, 1979) and that morphological changes in the tomato leaf can most probably also lead to an increased resistance against *Cladosporium fulvum* (De Wit, 1977; Lazarovits and Higgins, 1976a and 1976b). These findings suggest that chitinase and 1,3-ß-glucanase accumulation is most likely only part of a cascade of defence reactions eventually leading to resistance of tomato against *Cladosporium fulvum*.

Materials and methods

Fungus, plants and inoculation

Race 5 of *Cladosporium fulvum* (Cooke) (syn *Fulvia fulva* (Cooke) Cif) was grown on potato dextrose agar and a suspension of $5 \cdot 10^6$ conidia ml^{-1} was used to inoculate near isogenic lines Cf5 (susceptible to race 5) and Cf4 or Cf9 (resistant to race 5) of tomato (*Lycopersicon esculentum* Mill.) (De Wit, 1977; De Wit and Flach, 1979). Plants were grown in greenhouse conditions as described earlier (De Wit, 1977; De Wit and Flach, 1979).

Tissue processing for electron microscopy

Leaf samples (1 mm^2) were fixed by immersion in 1.0 % (w/v) paraformaldehyde and 0.7 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature under vacuum (20 kPa) and for 16 h at 4 °C, without the application of vacuum. The samples were subsequently washed with 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated in a graded ethanol series at -25°C and embedded in LR Gold at low temperature (-25°C) as described in the protocol provided by the manufacturer. The resin was polymerized under UV light at 360 nm. Ultrathin sections were cut on a LKB Ultrotome III with a Diatome diamond knife and collected on formvar coated golden grids (100 - 150 mesh).

Antibodies

Polyclonal antibodies, raised against the 26 kD extracellular chitinase and the 35 kD extracellular 1,3-ß-glucanase were prepared as described by Joosten and De Wit (Joosten and De Wit, 1989). With these antibodies we were able to detect both the intracellular and the extracellular isoforms of the native and denatured chitinases and 1,3-ß-glucanases (Joosten and De Wit, 1989).

Immunogold labelling

Tissue sections were preincubated on a drop of phosphate-buffered saline (pH 7.2) containing 1% (w/v) bovine serum albumin (PBS/BSA) for 30 min to prevent non-specific binding of the antibodies, followed by incubation with polyclonal antibodies, diluted 1:1000 in PBS/BSA, for two hrs. After rinsing the sections with 30 drops of PBS, they were incubated on a drop of colloidal gold-conjugated protein A (pAg) with gold particles of 7 nm (Van Lent and Verduin, 1986) in PBS/BSA (A_{520nm}=0.1) for 1.5 h. After rinsing with 20 drops of PBS the sections were fixed with 1 % (v/v) glutaraldehyde in PBS for 10 min, followed by rinsing with 20 drops of H_2O. The sections were stained with 2 % (w/v) uranylacetate in H_2O for 5 min and with 0.7 % (w/v) lead citrate in 0.05 N NaOH for 1 min. The sections were examined with a Philips CM 12 transmission electron microscope (TEM) at 80 kV.

Immunocytochemical controls

Specificity of the labelling was determined by the following control experiments: (1) incubation of the sections with serum obtained before rabbits were immunized, (2) incubation of the sections with chitinase and 1,3-ß-glucanase antibodies that had been saturated by preincubation with the corresponding antigen, (3) incubation of the sections with the colloidal gold-conjugated protein A only.

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chapter 4

Subcellular localization of PR-1 pathogenesis-related proteins in *Cladosporium fulvum*-infected tomato leaves

Jos P. Wubben and Pierre J.G.M. De Wit.

Abstract. As part of the defence response of tomato against infection by *Cladosporium fulvum*, the accumulation of pathogenesis-related (PR) proteins has been observed in the apoplast of infected leaves. Here we report on the spatial distribution of PR-1 proteins in *C. fulvum*-infected tomato leaves, studied by means of immunolocalization. These proteins accumulated several days earlier in an incompatible interaction between *C. fulvum* and tomato than in a compatible one. A role for PR-1 proteins in defence of tomato plants against *C. fulvum* is not known. Here we show that PR-1 proteins accumulate in the apoplast of *C. fulvum*-infected tomato leaves in extracellular material near the hyphae. Furthermore, degeneration of cytoplasm in leaf mesophyll cells was correlated with accumulation of PR-1 proteins. No spatial differences in PR-1 accumulation were observed when compatible and incompatible interactions between *C. fulvum* and tomato were compared. Accumulation of PR-1 proteins in the interaction between *C. fulvum* and tomato is most likely part of a complex array of plant defence responses which are not specifically aimed at prevention of infection by *C. fulvum*.

Introduction

The interaction between the fungal pathogen *Cladosporium fulvum* and tomato has been studied thoroughly at the cellular level. Detailed analyses using both light and electron microscopy, provided information on the infection process. *C. fulvum* penetrates tomato leaves through stomata in the lower epidermis and subsequently grows in the intercellular space between mesophyll cells. Penetration of mesophyll cells does not occur and formation of specialized feeding structures has never been observed (reviewed by Van den Ackerveken and De Wit, 1994).

The defence response of tomato against *C. fulvum* coincides with the production of numerous proteins which accumulate in infected leaf tissue (De Wit *et al.*, 1986; Joosten and De Wit, 1989; Joosten *et al.*, 1990). Several of these proteins were identified as pathogenesis-related (PR) proteins, accumulating 2-4 days earlier in incompatible than in compatible *C. fulvum*-tomato interactions. Some PR proteins possess antifungal activity, either by hydrolysing polymers that are present in fungal cell walls (1,3-ß-glucan, chitin) or by other, yet unknown mechanisms (Linthorst, 1991; Stintzi *et al.*, 1993). Several hydrolytic PR proteins (1,3-ß-glucanases and chitinases) have been purified from *C. fulvum*-infected tomato leaves (Joosten and De Wit, 1989; Joosten *et al.*, 1994; Van Kan *et al.*, 1992) and accumulation in time and space has been determined by immunolocalization studies (Wubben *et al.*, 1992). 1,3-ß-Glucanases and chitinases were found to accumulate near fungal hyphae in both compatible and incompatible interactions (Wubben *et al.*, 1992). Furthermore, accumulation of 1,3-ß-glucanases and chitinases has been observed in electron-dense material present in the intercellular space, and in vacuoles of mesophyll cells (Wubben *et al.*, 1992).

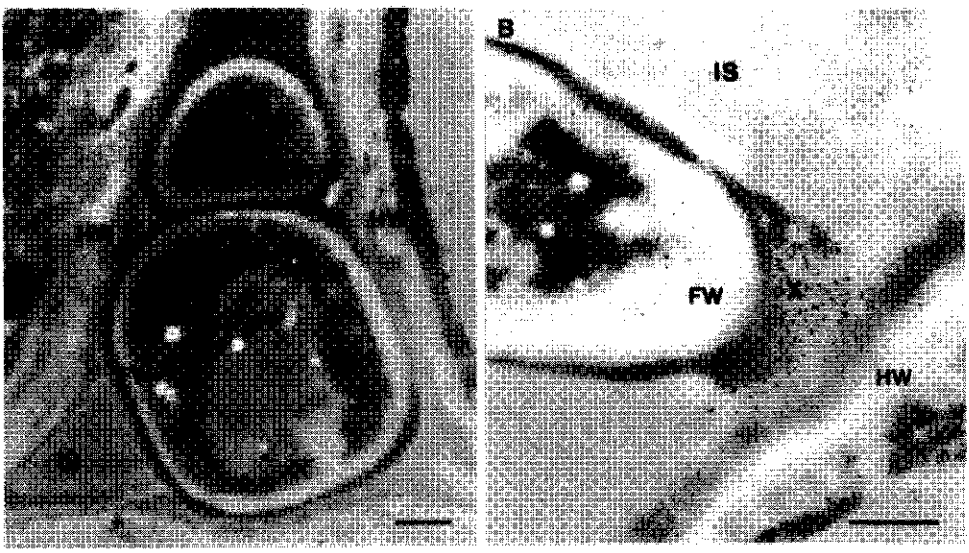

Fig. 1. Immunocytochemical localization of PR-1 pathogenesis-related proteins in thin sections of susceptible tomato leaves, infected by *Cladosporium fulvum*, obtained at 12 days post inoculation. Sections were incubated with antibodies raised against tomato PR-1b, followed by incubation with protein A-gold (7 nm). (A) Deposition of gold particles occurred in the intercellular space (IS) at material present between host cell walls (HW) and cell walls of the fungus (FW). (B) Strong deposition of gold particles is shown at material (X) in the intercellular space (IS) between plant cell wall (HW) and fungal cell wall (FW). Bar = 0.5 μm

Whether these potentially antifungal proteins play an essential role in the defence response of tomato against *C. fulvum* remains to be elucidated.

In addition to the presence of hydrolytic PR proteins, other host-encoded proteins belonging to the group of PR-1 proteins, were identified in the apoplast of *C. fulvum*-infected tomato leaves (De Wit and Van der Meer, 1986; Joosten *et al.*, 1990). The PR-1 proteins that have been purified from apoplastic fluid are PR-1a, formerly known as P4, and PR-1b, formerly known as P6 or P14 (Joosten *et al.*, 1990). A role for PR-1 proteins, which have no hydrolytic activity, in defence of tomato plants against *C. fulvum*, is not known. Sequence information on the purified PR-1 proteins and their encoding genes does not provide any clues on possible functions (Van Kan *et al.*, 1992). Antifungal activity of tomato PR-1 proteins against *Phytophthora infestans* has been observed in *in vitro* growth assays (Niderman *et al.*, 1993). Furthermore, transgenic tobacco plants, overexpressing tobacco PR-1a, revealed increased resistance against two oomycetous pathogens (Alexander *et al.*, 1993).

Here we report on the distribution of PR-1 proteins in *C. fulvum*-infected tomato leaves of both compatible and incompatible interactions, as studied by immunolocalization.

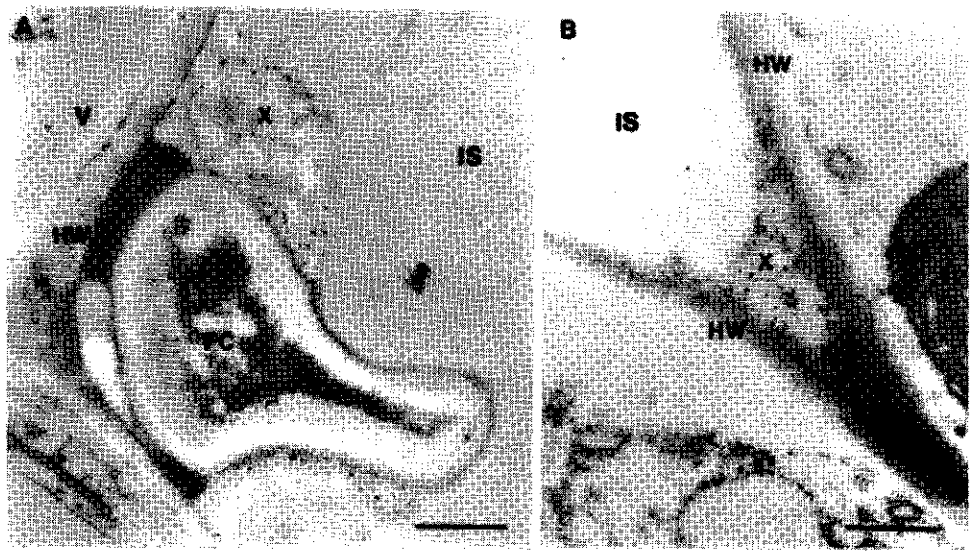

Fig. 2. Immunocytochemical localization of PR-1 pathogenesis-related proteins in thin sections of resistant tomato leaves, infected by *Cladosporium fulvum*, obtained at 12 days post inoculation. Sections were incubated with antibodies raised against tomato PR-1b, followed by incubation with protein A-gold (7 nm). (A) Clear deposition of gold particles is visible in material (X) near fungal hyphae (FC) in the intercellular space (IS) of the infected leaf. No labelling was observed inside fungal hyphae, in or at the host cell wall (HW) or inside vacuoles of the host (V). (B) Extracellular material (X) at three-way junctions between cell walls (HW) of two mesophyll cells contained some gold particles. Chloroplasts (C) in the mesophyll cells were free of labelling. Bar = 0.5 μm

Implications of these results towards a role for PR-1 proteins in defence of tomato against *C. fulvum* will be discussed.

Results

Immunolocalization of PR-1 proteins in C. fulvum*-infected tomato leaves*

Polyclonal antibodies, previously obtained from rabbits immunized with purified tomato PR-1b (Joosten *et al.*, 1990), were used for localization of PR-1 proteins in *C. fulvum*-infected tomato leaves. The antibodies were shown to detect both PR-1a and PR-1b (Joosten *et al.*, 1990). Ultrathin sections of *C. fulvum*-inoculated tomato leaves were incubated with PR-1b antibodies, followed by protein A-gold labelling. PR-1 proteins were found to accumulate in the intercellular space of infected leaves near extracellular material, either around fungal hyphae (Fig. 1, A and B and Fig. 2, A) or at three-way junctions between tomato mesophyll cells (Fig. 2, B) in both a compatible (Fig. 1) and an incompatible interaction between tomato and *C. fulvum* (Fig. 2). No PR-1 proteins have been observed in protein aggregates

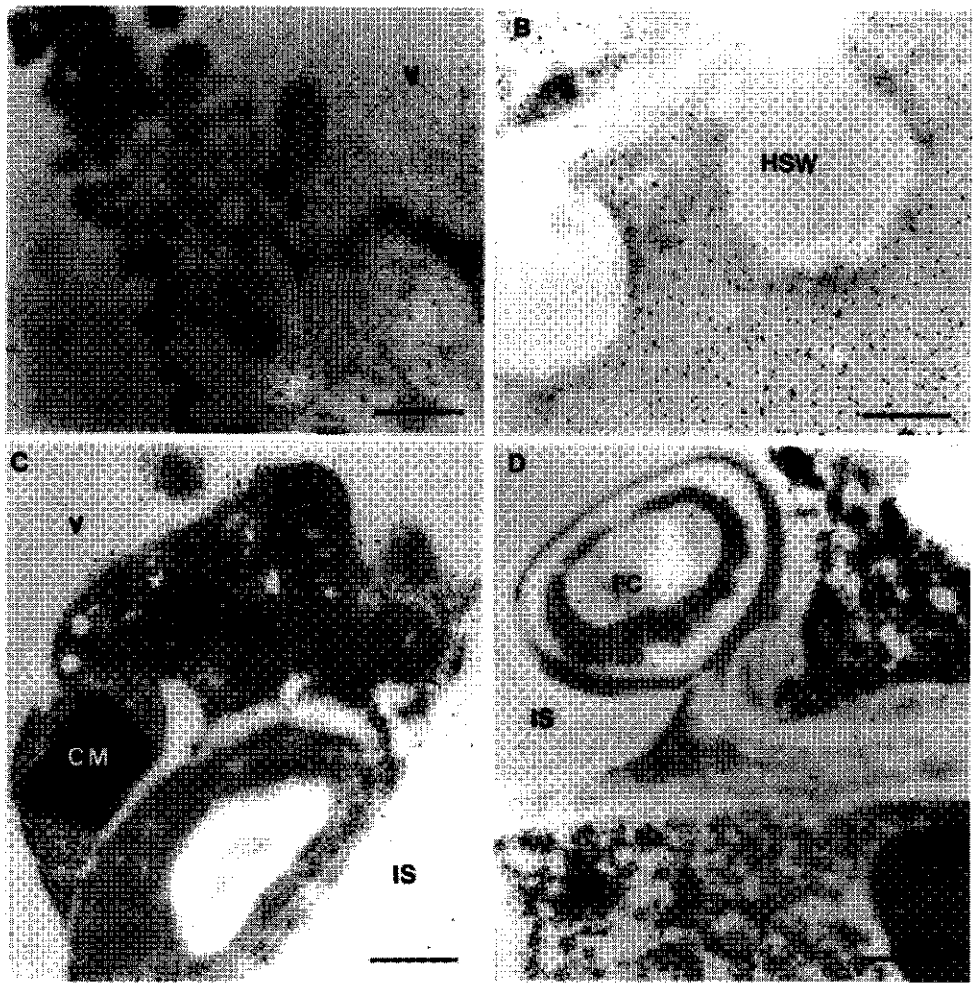

Fig. 3. Immunocytochemical localization of PR-1 pathogenesis-related proteins in thin sections of resistant (A and B) and susceptible (C and D) tomato leaves, infected by *Cladosporium fulvum*, obtained at 12 days post inoculation. Sections were incubated with antibodies raised against tomato PR-1b, followed by incubation with protein A-gold (7 nm). (A) Protein aggregates (PA) in vacuoles (V) of *C. fulvum*-infected tomato leaves of an incompatible interaction revealed no labelling with antibodies raised against tomato PR-1b. (B) Clear deposition of gold particles is visible in vessels of vascular tissue whereas host secondary walls (HSW) inside vessels were free of labelling. (C) Several mesophyll cells of *C. fulvum*-infected tomato leaves revealed a clear deposition of gold particles, as shown for susceptible tomato leaves obtained at 12 days post inoculation. No labelling was found in crystalline microbodies (CM), mitochondria (M), chloroplasts (C) or vacuoles (V) of mesophyll cells. (D) Mesophyll cells containing disturbed cytoplasm (DC) showed substantial gold deposition, while no labelling was found in fungal cells (FC) or intercellular space (IS) of tomato leaves. Bar = 0.5 μm

in vacuoles of infected tomato leaves (Fig. 3, A). Accumulation has been observed in vascular tissue (Fig. 3, B) as well as in cytoplasm of intact (Fig. 3, C) or degenerated

mesophyll cells (Fig. 3, D). Again no significant differences in spatial distribution of PR-1 proteins between compatible and incompatible interactions have been observed.

Discussion

Here we describe the spatial distribution of PR-1 proteins in *C. fulvum*-infected tomato leaves. The accumulation of PR-1 proteins in the apoplast of *C. fulvum*-infected tomato leaves was associated with different extracellular material, either present around fungal hyphae, or at three-way junctions between mesophyll cells in both compatible and incompatible interactions. The spatial distribution of the extracellular PR-1 proteins gave no indication on a possible involvement in the defence response of tomato against *C. fulvum*. In extracellular material as observed in Figure 1B, in addition to PR-1, an accumulation of the extracellular fungal proteins ECP1 and ECP2 has been observed (Wubben *et al.*, 1994). A function of this extracellular matrix, containing plant and fungal proteins, in the infection process is unknown.

PR-1 accumulation was observed in vascular bundles and in the cytoplasm of degenerated mesophyll cells. Occurrence of PR-1 proteins in mesophyll cells containing degenerated cytoplasm has been reported before by Vera *et al.* (1988). Since this observation was made in non-infected tissue, it was suggested that PR-1 proteins are involved in a naturally-induced cell-degenerative process.

Vacuolar accumulation in mesophyll cells of *C. fulvum*-infected tomato leaves as observed with chitinase and 1,3-ß-glucanase, was not detected for PR-1. The absence of labelling in vacuolar protein aggregates, indicates that either PR-1 proteins do not occur in vacuoles of *C. fulvum*-infected tomato leaves or that antibodies specific for apoplastic PR-1b do not cross-react with the existing vacuolar isoforms of PR-1 proteins. Vacuolar accumulation of PR-1 was found in viroid-infected tomato plants indicating that these PR-1 isoforms do exist in tomato (Vera *et al.*, 1989).

PR-1 accumulation, as determined by immunolocalization, has been described for several plant-pathogen interactions. Tomato roots infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici* revealed a clear PR-1 accumulation in host cell walls and secondary cell wall thickenings (Benhamou *et al.*, 1991). No accumulation at fungal cell walls has been observed. PR-1 accumulation near host cell walls has also been found in tobacco root tissue infected by *Chalara elegans* (Tahiri-Alaoui *et al.*, 1993). It has been suggested that PR-1 proteins play a role in cell wall modifications. These modifications could, in combination with the deposition of hydroxyproline-rich glycoproteins and pectic substances, result in strengthening of the cell wall, which functions as a mechanical barrier against pathogens (Benhamou *et al.*, 1991). However, clear differences in spatial distribution of PR-1 accumulation between compatible and incompatible plant-fungus interactions have not been

described (Benhamou *et al.*, 1991; Tahiri-Alaoui *et al.*, 1993). In tobacco roots infected by *C. elegans* a strong association of PR-1 proteins with fungal cell walls has been observed. However, a direct effect of tobacco PR-1 on growth of *C. elegans in vitro*, has not been found (Tahiri-Alaoui *et al.*, 1993).

Direct *in vitro* antifungal activity of tomato PR-1 proteins has been obtained against *Phytophthora infestans* (Niderman *et al.*, 1993). Niderman and coworkers found an inhibitory effect on zoospores of *P. infestans* at concentrations of PR-1 ranging from 10 to 40 ppm. In addition, PR-1-overexpressing transgenic tobacco plants showed reduced susceptibility towards two oomycetous pathogens (Alexander *et al.*, 1993). These results indicate that PR-1 proteins can play a role in defence of plants against oomycetous pathogens. The mechanism by which PR-1 proteins cause increased resistance is not clear. Studies on the PR-1 distribution in PR-1-overexpressing transgenic tobacco plants, following inoculation with these pathogens, can provide information on the mechanism of resistance effected by PR-1 proteins.

A role for PR-1 accumulation in the interaction between *C. fulvum* and tomato remains to be elucidated. Most likely, accumulation of PR-1 is part of an array of plant defence responses, not primarily aimed at inhibition of growth of *C. fulvum*. Genetic evidence for the existence of a programmed defence response in plants was described recently for mutant Arabidopsis lines which revealed spontaneous hypersensitive response-like symptoms. These symptoms correlated with other phenomena observed in plant defence responses such as modifications of the plant cell wall, accumulation of the signal molecule salicylic acid and accumulation of defence-related gene transcripts encoding different PR proteins including PR-1 (Dietrich *et al.*,1994; Greenberg *et al.*,1994).

Materials and methods

Fungus, plants and inoculation

Race 5 of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) was grown on potato dextrose agar and a suspension of $5 \cdot 10^6$ conidia ml^{-1}, obtained from 7 to 10-day-old cultures, was used to inoculate near-isogenic lines Cf5 (susceptible to race 5) and Cf4 (resistant to race 5) of tomato cultivar Moneymaker (*Lycopersicon esculentum* Mill.) (De Wit, 1977; De Wit and Flach, 1979). Plants were grown as described before (De Wit, 1977; De Wit and Flach, 1979).

Tissue processing for electron microscopy

Leaf samples (1 mm^2) were fixed by immersion in 1.0 % (w/v) paraformaldehyde and 0.7 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature under vacuum (20 kPa). Subsequently, tissue was incubated for 16 h at 4 °C

without the application of vacuum. Leaf samples were washed with 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated in a graded ethanol series at -25°C and embedded in LR Gold at low temperature (-25°C). Polymerization of LR Gold resin containing the leaf samples was performed with UV light at 360 nm. Ultrathin sections (60 - 80 nm) were cut on a LKB Ultrotome III with a Diatome microtome knife and collected on golden, formvar coated grids (100 - 150 mesh).

Preparation and characterization of the antibodies

PR-1b antibodies were obtained by immunizing rabbits with purified tomato PR-1b as described before (Joosten *et al.*, 1990). Western blotting revealed that PR-1b antibodies detected both PR-1a and PR-1b in apoplastic fluid of *C. fulvum*-infected tomato leaves (Joosten *et al.*, 1990).

Immunolocalization of PR-1b

Tissue sections of LR Gold embedded leaf material were preincubated on a drop of phosphate-buffered saline (pH 7.2), containing 1% (w/v) bovine serum albumin (PBS/BSA) for 30 min, followed by incubation with polyclonal PR-1b antibodies, diluted 1:1000 in PBS/BSA for 2 h. Subsequently, sections were incubated on a drop of colloidal gold-conjugated protein A with gold particles of 7 nm (Van Lent and Verduin, 1986) in PBS/BSA (A_{520nm}=0.1) for 1.5 h. The sections were stained with 2 % (w/v) uranylacetate in H_2O for 5 min and with 0.7 % (w/v) lead citrate in 0.05 N NaOH for 2 min and subsequently examined with a Philips CM 12 transmission electron microscope at 80 kV.

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chapter 5

Distribution of extracellular matrix components of fungus and plant in the *Cladosporium fulvum*-tomato interaction

Jos P. Wubben and Pierre J.G.M. De Wit.

Abstract. Hydrolytic cell wall-degrading enzymes play several roles in plant-pathogen interactions. These enzymes, produced either by the pathogen or the defending host plant can facilitate fungal penetration, exhibit antifungal activity or can indirectly induce defence responses in plants by oligosaccharides generated. In the interaction between the fungal pathogen *Cladosporium fulvum* and tomato, accumulation of several hydrolytic enzymes has been reported which potentially have an influence on the infection process. Further investigation on a possible role of hydrolytic enzymes in the interaction between *C. fulvum* and tomato requires detailed studies on the cellular distribution of substrates for these enzymes by immunolocalization, as described in this paper. Pectolytic activity, as studied indirectly by distribution of unesterified pectins and methylesterified pectins in the extracellular matrix of infected tomato leaves in a compatible interaction with *C. fulvum*, could not be observed. Furthermore, chitinases produced by the plant upon infection by *C. fulvum*, have no significant effect on distribution and amount of chitin in the cell wall of the invading hyphae. These enzymes probably have no access to the chitin present in the cell walls of *C. fulvum* growing *in planta*.

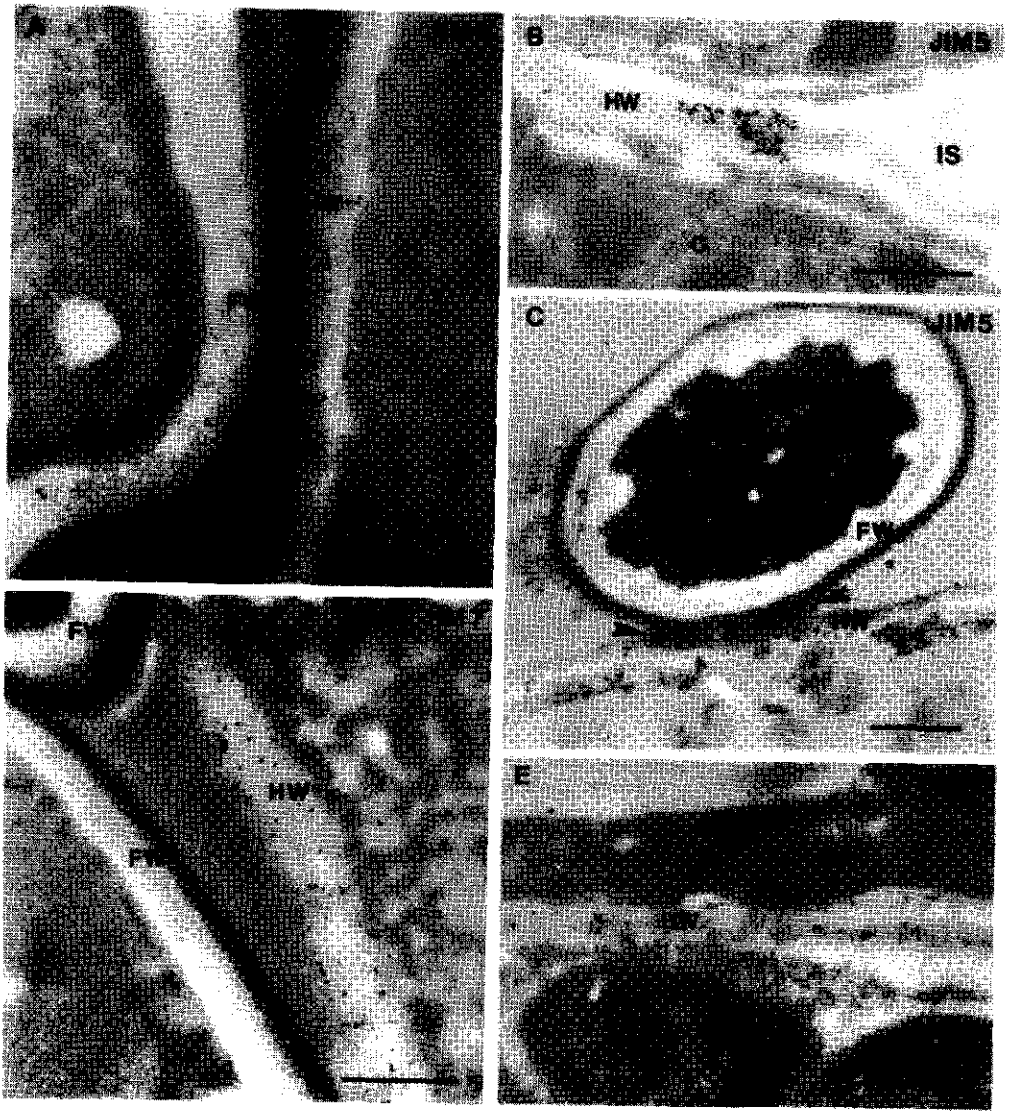
Introduction

Hydrolytic cell wall-degrading enzymes can play various roles in plant-pathogen interactions. Enzymes of fungal origin might be involved in infection processes, either in recognition and adhesion events (Nicholson and Epstein, 1991), or in degradation of plant cell walls prior to fungal penetration (Köller, 1991). These enzymes can release oligogalacturonides or xyloglucan oligosaccharides from plant cell walls, which induce various defence responses in plants (Albersheim *et al.*, 1992). Hydrolytic enzymes produced by plants in response to pathogen invasion can hydrolyse fungal cell walls. If lysis of fungal cell walls occurs, it can result in growth inhibition and prevention of further colonization of the host (Schlumbaum *et al.*, 1986). Hydrolysis of fungal cell walls can also result in the release of oligoglucoside elicitors which might also induce defence responses in plants (Albersheim *et al.*, 1992; Keen and Yoshikawa, 1983).

Thorough investigation of the interaction between the fungal pathogen *Cladosporium fulvum* and tomato has resulted in the isolation and characterization of several hydrolytic enzymes of plant origin, which accumulate in the host during pathogenesis (Joosten and De Wit, 1989). These plant enzymes are potentially able to hydrolyse fungal wall components such as chitin and 1,3-ß-glucan. *In vitro* antifungal activity towards *Trichoderma viride* has been observed for some of the chitinases and 1,3-ß-glucanases isolated from leaves of tomato in a compatible interaction with *C. fulvum* (Joosten *et al.*, 1994). Surprisingly, *in vitro* growth of *C. fulvum* could not be inhibited with partially purified isoforms of chitinases and 1,3-ß-glucanases (Joosten *et al.*, 1994). Immunolocalization of these enzymes in *C. fulvum*-infected tomato leaves revealed accumulation of chitinases and 1,3-ß-glucanases near hyphae

of *C. fulvum*, in the apoplast of both compatible and incompatible interactions (Wubben *et al.*, 1992).

Only limited data were obtained on the accumulation of plant cell wall-degrading enzymes of either plant or fungal origin. A slight increase in plant cell wall-hydrolysing activity has been found in apoplastic fluid of *C. fulvum*-infected tomato leaves for both compatible and incompatible interactions, when compared to healthy controls (Aldington and Fry, 1992).



Further investigations on a role for plant cell wall-degrading enzymes in the interaction between *C. fulvum* and tomato require detailed studies on the cellular distribution of possible substrates for these enzymes by immunolocalization. Probes were used to study alterations in pectin distribution in plant cell walls. Furthermore, in order to assess whether chitinases play a role in the interaction between *C. fulvum* and tomato, the distribution of chitin in the cell wall of *C. fulvum* growing either *in vitro* or *in planta*, was studied. A possible role of cell wall-hydrolysing enzymes in the interaction between *C. fulvum* and tomato will be discussed.

Results

Distribution of pectin in the extracellular matrix of C. fulvum-*infected tomato leaves*

Involvement of pectolytic enzymes in the interaction between *C. fulvum* and tomato has been investigated indirectly by monitoring alterations in pectin distribution in the extracellular matrix of healthy and infected plant tissue by immunolocalization. In these studies, monoclonal antibodies specific for unesterified pectin (JIM 5) and methylesterified pectin (JIM 7), were used. Gold-conjugated secondary antibodies were used to detect the JIM5 and JIM7 antibodies. Unesterified pectin was abundantly present in the outer-layer of the plant extracellular matrix in both tomato leaf tissue in a compatible interaction with *C. fulvum* (Fig. 1, A) and healthy leaf tissue (data not shown), and near three-way junctions between mesophyll cells (Fig. 1, B). Furthermore, electron-dense, extracellular material present between fungal and host cell walls exhibited labelling with antibodies specific for unesterified pectin (Fig. 1, C). Methylesterified pectin occurred throughout the plant extracellular matrix (Fig. 1, D and E). The cellular distribution of unesterified pectin and methylesterified pectin in the extracellular matrix of mesophyll cells from infected tomato leaves in a compatible interaction with *C. fulvum* did not differ significantly from the distribution in infected tomato leaves.

Fig. 1. Immunocytochemical localization of unesterified pectin and methylesterified pectin in healthy and *Cladosporium fulvum*-infected tomato leaf tissue. Sections were incubated with rat monoclonal antibodies, specific for unesterified pectin (JIM5) (A, B and C) and methylesterified pectin (JIM7) (D and E), followed by incubation with colloidal gold-conjugated goat-anti-rat antibodies (5 nm). Shown are healthy (B) and *C. fulvum*-infected tomato leaf tissue of a compatible interaction (Cf5/race5), obtained 12 days after inoculation (A and C) and incubated with JIM 5 antibodies. (A) Clear deposition of gold particles is observed at the outer layer of the plant cell wall (HW) near the fungal cell wall (FW) and (B) at three-way-junctions between tomato mesophyll cells near the intercellular space (IS). Intracellular chloroplasts (C) were free of labelling. (C) Extracellular material (X) between the cell wall of the host (HW) and the cell wall of the fungus (FW) revealed sparse labelling with the JIM 5 antibodies (arrowheads). Healthy (E) and *C. fulvum*-infected tomato leaf tissue of a compatible interaction (Cf5/race5), obtained 12 days after inoculation (D) and incubated with JIM 7 antibodies (D and E). (D) Dispersed distribution of gold particles is visible in the cell wall of the host (HW) attached to the fungal wall (FW). No labelling was observed at the fungal cell wall (FW). (E) In between tomato mesophyll cells a diffuse labelling was observed throughout the cell walls (HW). Bar = 0.5 μm

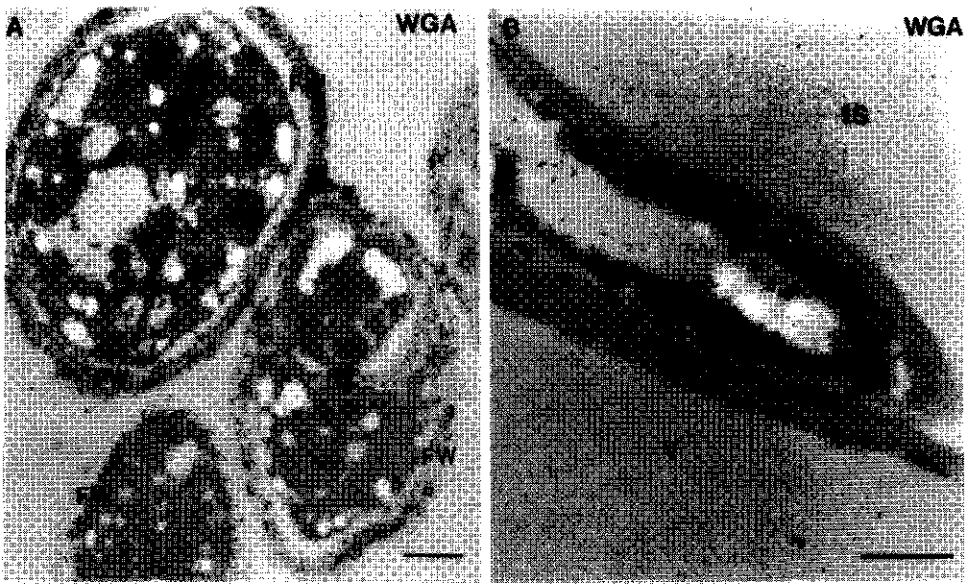


Fig. 2. Immunocytochemical localization of chitin in cell walls of *Cladosporium fulvum* grown *in vitro* (A) and *in planta* (B). Wheat germ agglutinin (WGA) was used to detect chitin in the cell walls of *C. fulvum*. (A) WGA labelling of hyphae of *C. fulvum* obtained from liquid shake cultures occurred in the cell walls around the fungus (FW) or at the inner walls (IW) within the hyphae (septa). (B) Tomato leaf tissue of a compatible interaction (Cf5/race 5), obtained 12 days post inoculation. Labelling with WGA was found primarily in the wall (FW) of hyphae growing in the intercellular space (IS). No labelling was found at the host cell wall (HW) and vacuole (V) of mesophyll cells. Bar = 0.5 μm

Distribution of chitin in cell walls of C. fulvum *grown* in vitro *and* in planta

Wheat germ agglutinin (WGA), a lectin with N-acetylglucosamine-binding properties, was used as a probe to localize chitin in fungal cell walls in tomato leaf tissue in a compatible interaction with *C. fulvum*. WGA-specific antibodies were used to detect bound WGA and these were labelled with colloidal gold-conjugated protein A. Chitin distribution in hyphae of *C. fulvum* was found to be evenly spread in the inner layer of fungal cell walls (Fig. 2, A and B). Chitin was hardly observed in the electron-dense outer layer of the fungal cell wall. The intensity of gold particles in fungal hyphae grown *in vitro* (Fig. 2, A) and *in planta* (Fig. 2, B) was similar. No chitin degradation of fungal cell walls in compatible *C. fulvum*-tomato interactions was observed (Fig. 2, B).

Discussion

The role of plant cell wall-hydrolysing enzymes in the interaction between *C. fulvum* and tomato is likely to be of minor importance. Pectolytic activity, as studied indirectly by the

distribution of unesterified pectin and methylesterified pectin in the plant extracellular matrix of *C. fulvum*-infected tomato leaves, has not been observed. Growth of *C. fulvum* is strictly confined to the apoplast of tomato leaves, and therefore there is no need for the fungus to degrade plant cell walls. However, Aldington and Fry (1992) have been able to detect an increase in cell wall-degrading activity in apoplastic fluid of C. fulvum-infected tomato leaves by using a sensitive assay with radio-labelled plant cell walls.

Both unesterified pectin and arabinogalactan proteins (results not shown) were detected in low quantities in electron-dense extracellular material near the interface of fungal and host cell walls. We already have reported on the accumulation of fungal (Wubben *et al.*, 1994) and plant proteins (Wubben *et al.*, 1992) in electron-dense material around fungal hyphae in the intercellular space of infected tomato leaves. A role for this extracellular material in the interaction between *C. fulvum* and tomato is yet unknown. The occurrence of unesterified pectin in extracellular material near fungal hyphae might be a reflection of pectolytic activity of plant or fungal origin. The pectin fragments released from the plant cell wall can subsequently accumulate at the extracellular matrix between fungus and host.

A clear disturbance in pectin distribution in plant cell walls has been observed in bean and tomato upon infection by *Colletotrichum lindemuthianum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici*, respectively (Benhamou *et al.*, 1990a and 1991). These observations were made using Aplysia gonad lectin, a polygalacturonic acid-binding lectin. Accumulation of polygalacturonic acid-containing molecules has been observed in altered phloem cells of *F. oxysporum*-infected tomato roots (Benhamou *et al.*, 1990a). Furthermore, in the interaction between bean and *C. lindemuthianum*, the accumulation of pectin molecules has been observed at specific sites, such as intercellular spaces and aggregated cytoplasm of host cells. The accumulation of pectin molecules at strategic sites in infected leaves was thought to support their involvement in the plant defence response upon fungal attack (Benhamou *et al.*, 1990a and 1991).

Chitinases, produced by the infected tomato plants in a compatible interaction with *C. fulvum*, do not seem to have an effect on the distribution and amount of chitin present in the cell wall of the fungus. Additional evidence for a minor role, if any, of tomato chitinases in defence against *C. fulvum*, was found in *in vitro* growth assays. Growth of *C. fulvum*, in contrast to *Trichoderma viride*, is not affected by high concentrations of tomato chitinases. Our results indicate that chitin in cell walls of *C. fulvum* is probably not accessible to these hydrolytic enzymes.

Chitin distribution in fungal cell walls as determined by means of WGA labelling has been studied in several plant-pathogen interactions. For *F. oxysporum* f. sp. *radicis lycopersici*-infected tomato roots, it has been reported that WGA labelling in fungal cell walls in close contact with tomato root cells was lower than the WGA labelling observed in free growing hyphae *in planta* (Benhamou *et al.*, 1990b). However, no differential WGA labelling

of hyphae of *F. oxysporum* has been observed in compatible and incompatible interactions. Extensive chitin degradation in the cell walls of *Rhizoctonia solani* correlating with inhibition of growth of this fungus in the presence of increasing concentrations of a bean chitinase, has been reported to occur either in *in vitro* antifungal assays (Benhamou *et al.*, 1993a) or in *R. solani*-infected chitinase-overexpressing Canola plants (Benhamou *et al.*, 1993b). These observations suggest that plant chitinases can play a role in defence against fungi, such as *F. oxysporum* and *R. solani*, but most likely not against *C. fulvum*. Other components of the defence response are involved in resistance of tomato against *C. fulvum*. Probably the hypersensitive response, a rapid cell death which takes place in incompatible interactions between *C. fulvum* and tomato, in concert with other early defense responses, such as the release of active oxygen species (Vera-Estrella *et al.*, 1992), play a major role in the arrest of fungal growth in resistant tomato plants.

Materials and methods

Fungus, plants and inoculation

Race 5 of *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) was subcultured on potato dextrose agar and a suspension of $5 \cdot 10^6$ conidia ml^{-1}, obtained from 7 to 10-day-old cultures, was used to inoculate near-isogenic lines Cf5 (susceptible to race 5) and Cf4 (resistant to race 5) of tomato cultivar Moneymaker (*Lycopersicon esculentum* Mill.) (De Wit 1977; De Wit and Flach 1979). Plants were grown as described before (De Wit 1977; De Wit and Flach 1979). *C. fulvum* was cultured in liquid shake culture as described before (De Wit and Roseboom, 1980).

Tissue processing for electron microscopy

Leaf samples (1 mm^2) or mycelium of *C. fulvum* grown in liquid shake culture were fixed by immersion in 1.0 % (w/v) paraformaldehyde and 0.7 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature, under vacuum (20 kPa) and for 16 h at 4 °C, without application of vacuum. Samples were subsequently washed with 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated in a graded ethanol series and embedded in LR Gold at low temperature (-25°C) or in Epon 812 at room temperature. Polymerization of LR Gold resin was performed with UV light at 360 nm. Epon 812 was polymerized by incubation at 60 °C. Ultrathin sections (60 - 80 nm) were cut on a LKB Ultrotome III with a Diatome microtome knife and were collected on golden, formvar-coated grids (100 - 150 mesh).

Antibodies used for detection of extracellular matrix components

Monoclonal antibodies specific for plant extracellular matrix components were kindly provided by Dr M. McCann (John Innes Institute, Department of Cell Biology, Norwich). These monoclonal antibodies were obtained from rats immunized with carrot protoplasts (Knox *et al.*, 1990; VandenBosch *et al.*, 1989). The monoclonal antibodies were specific for unesterified pectin (JIM5) and methylesterified pectin (JIM7) (Knox *et al.*, 1990; VandenBosch *et al.*, 1989).

Immunogold detection of extracellular matrix components

Monoclonal antibodies JIM 5 and JIM 7 were used for localization of pectin in *C. fulvum*-infected tomato leaves. Tissue sections of LR Gold-embedded leaf material were preincubated on a drop of phosphate-buffered saline (pH 7.2) containing 1% (w/v) bovine serum albumin (PBS/BSA) for 30 min, followed by incubation with 2 % (v/v) hybridoma culture supernatant solution of the monoclonal antibodies in PBS/BSA, for 2 h. Subsequently, sections were incubated on a drop of colloidal gold-conjugated goat-anti-rat antibodies (Aurion, Wageningen, the Netherlands), with gold particles of 5 nm, in PBS/BSA (A_{520nm}=0.1) for 1.5 h. The sections were stained with 2 % (w/v) uranylacetate in H_2O for 5 min and with 0.7 % (w/v) lead citrate in 0.05 N NaOH for 2 min and subsequently examined with a Philips CM 12 transmission electron microscope at 80 kV.

Immunocytochemical localization of N-acetylglucosamine residues

For the N-acetylglucosamine localization, tissue sections of Epon-embedded fungal material were incubated on a drop of PBS/BSA for 30 min. N-acetylglucosamine localization was subsequently determined by incubation of the sections on a drop of PBS containing 25 μg ml^{-1} wheat germ agglutinin (WGA, Sigma), for 2 h. WGA was subsequently detected by incubation with anti-WGA antibodies (Sigma), diluted 1:500 in PBS, for 1 h, followed by detection with colloidal gold-conjugated protein A with gold particles of 7 nm (Van Lent and Verduin, 1986) for 1.5 h. Epon sections were stained with 2 % (w/v) uranyl acetate in H_2O for 10 min, followed by 0.7 % (w/v) lead citrate in 0.05 N NaOH for 5 min and subsequently examined with a Philips CM12 transmission electron microscope at 80 kV.

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chapter 6

Differential induction of PR protein gene expression in tomato by *Cladosporium fulvum* and its race-specific elicitors

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submitted

Abstract. Accumulation of pathogenesis-related proteins is an important feature of plant defence responses upon infection by pathogens. A role has been suggested in resistance-gene-mediated defence against pathogens, as well as in local and systemic acquired resistance. In the interaction between *Cladosporium fulvum* and tomato, resistance against the fungus correlates with early accumulation of apoplastic chitinase and 1,3-ß-glucanase in the inoculated tomato leaves. The early transcript accumulation of genes encoding apoplastic, acidic isoforms of the hydrolytic enzymes, revealed the same temporal differences between compatible and incompatible interactions as observed at the protein level by western blotting. With vacuolar, basic isoforms, however, early gene transcript accumulation was observed in both incompatible and compatible interactions. Here we report on the tissue-specific expression of genes encoding these hydrolytic enzymes as studied by means of *in situ* hybridization. Only temporal differences in gene transcript accumulation were observed for each isoform studied. Expression of the acidic chitinase and 1,3-ß-glucanase genes was observed primarily near leaf vascular tissue and near the epidermis, whereas expression of the basic chitinase and 1,3-ß-glucanase genes was not limited to particular tissues. No preferential accumulation of transcripts near penetrating fungal hyphae was observed in compatible or incompatible interactions. Injection of purified race-specific elicitors, AVR4 and AVR9, in tomato genotypes Cf4 and Cf9, respectively, gave specific induction of primarily acidic chitinase and 1,3-ß-glucanase gene expression. The induction, only observed in resistant genotypes, correlates well with the difference in gene expression as previously observed between compatible and incompatible *C. fulvum*-tomato interactions.

Introduction

Accumulation of pathogenesis-related (PR) proteins is an important feature of plant defence responses upon infection by pathogens. To elucidate the function of PR proteins in defence of plants against fungal pathogens first attempts were mainly aimed at a search for *in vitro* antifungal activity of purified PR proteins (Mauch *et al.*, 1988b; Schlumbaum *et al.*, 1986). Most groups of PR proteins displayed *in vitro* antifungal activity against a limited number of fungi (Hejgaard *et al.*, 1992; Mauch *et al.*, 1988b; Ponstein *et al.*, 1994; Vigers *et al.*, 1991; Woloshuk *et al.*, 1991). Furthermore, combined application of different PR proteins in an *in vitro* assay resulted in synergistic increase in antifungal activity (Mauch *et al.*, 1988b; Ponstein *et al.*, 1994; Sela-Buurlage *et al.*, 1993). Transgenic plants, constitutively expressing PR protein genes, gained increased resistance levels against a number of fungal pathogens (Alexander *et al.*, 1993; Broglie *et al.*, 1991; Liu *et al.*, 1994; Van den Elzen *et al.*, 1993). Besides resistance gene-mediated defence, local or systemic acquired resistance is an other, indirect way of defence against invading pathogens (Chester, 1933; Kuć, 1982). Acquired resistance is correlated with accumulation of several PR proteins in parts of the immunized plant (Pan *et al.*, 1991; Pan *et al.*, 1992; Ward *et al.*, 1991). This suggests that

the induction of PR proteins may play a role in active resistance of plants against fungal pathogens.

Our research involves detailed studies on the PR proteins that are induced in the interaction between the biotrophic, fungal pathogen *Cladosporium fulvum* and tomato. This interaction has been shown to comply with the gene-for-gene model; race-specific elicitors, which are the products of fungal avirulence genes, are the only factors inducing a hypersensitive response (HR) in tomato genotypes carrying the corresponding resistance genes (Joosten *et al.*, 1994; Van den Ackerveken *et al.*, 1992; Van Kan *et al.*, 1991). HR subsequently leads to activation of other defence responses, such as callose formation, phytoalexin production, and accumulation of PR proteins, either in vacuoles or in the apoplast, which is colonized by the fungus (recently reviewed by Van den Ackerveken and De Wit, 1994).

PR protein accumulation in apoplastic fluids of *C. fulvum*-inoculated tomato leaves occurred 2-4 days earlier in incompatible interactions than in compatible ones (De Wit and Van der Meer, 1986). Some of the apoplastic PR proteins that accumulated early in the incompatible interaction were identified as chitinases and 1,3-ß-glucanases (Joosten *et al.*, 1989). Immunolocalization experiments revealed that chitinases and 1,3-ß-glucanases accumulated in the intercellular space near unidentified, electron-dense material as well as near fungal hyphae (Wubben *et al.*, 1992). Furthermore, local accumulation of chitinase and 1,3-ß-glucanase was observed near stomatal guard cells (Wubben *et al.*, 1993) and in protein aggregates present in vacuoles of mesophyll cells (Wubben *et al.*, 1992). Northern blot analysis revealed that expression of acidic, extracellular chitinase and 1,3-ß-glucanase genes occurred 4 days earlier in incompatible interactions than in compatible ones (Danhash *et al.*, 1993; Van Kan *et al.*, 1992). However, for basic chitinase and 1,3-ß-glucanase, timing of gene expression was shown to be similar in compatible and incompatible interactions (Danhash *et al.*, 1993; Van Kan *et al.*, 1992).

Here we report on the spatial distribution of expression of chitinase and 1,3-ß-glucanase genes in *C. fulvum*-infected tomato leaf tissue, as studied by *in situ* hybridization. These studies were carried out as an extension of northern blot analysis (Danhash *et al.*, 1993; Van Kan *et al.*, 1992), in order to explain and extent some of the differences observed between compatible and incompatible *C. fulvum*-tomato interactions. Since race-specific elicitors of *C. fulvum* are responsible for induction of successful defence in resistant tomato genotypes (Joosten *et al.*, 1994; Van den Ackerveken *et al.*, 1992) accumulation of chitinase and 1,3-ß-glucanase mRNAs was studied by northern analyses after injection of purified elicitors in different tomato genotypes. The possible role of chitinase and 1,3-ß-glucanase in resistance of tomato against *C. fulvum* will be discussed.

Results

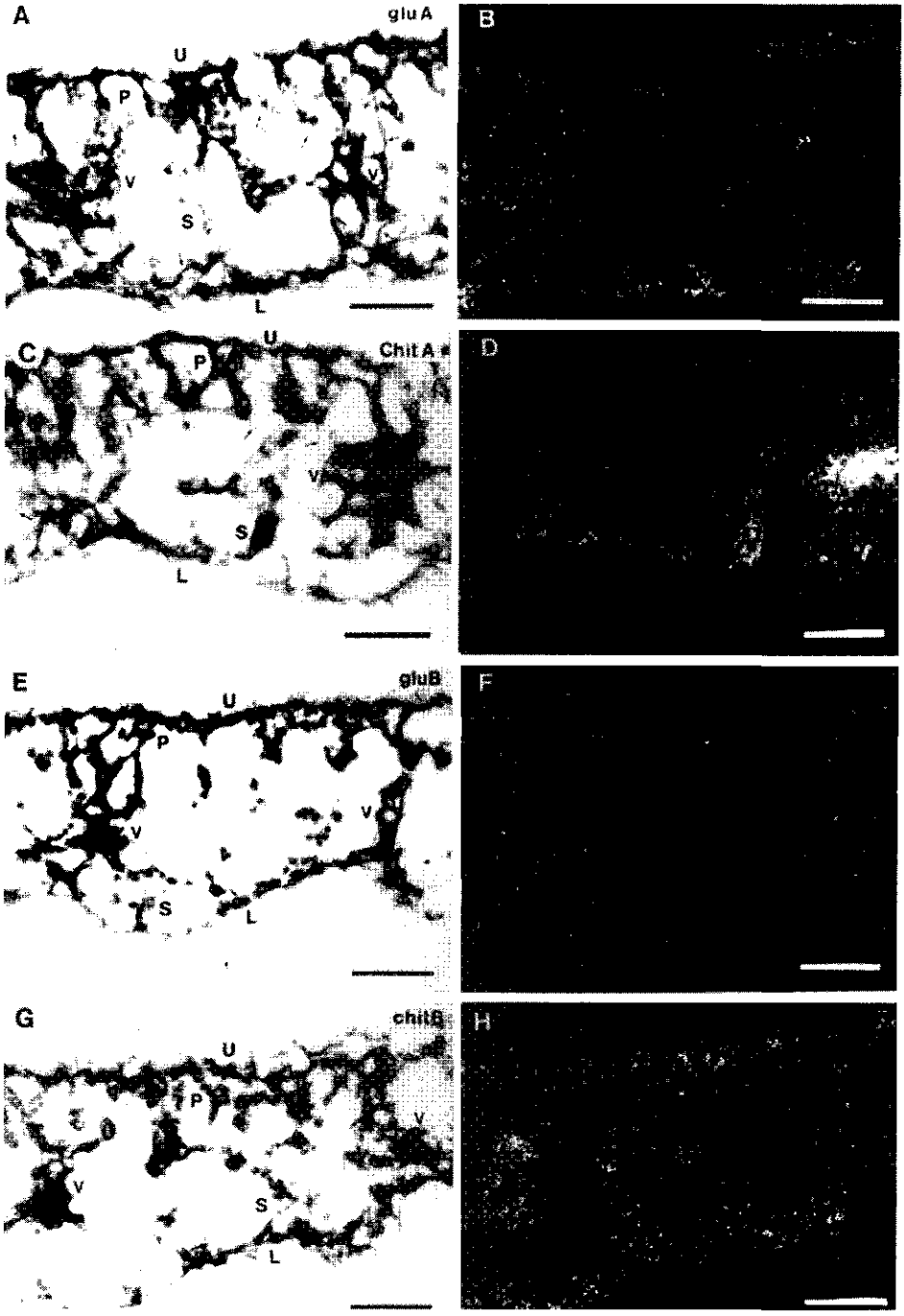
In situ hybridization in sections from C. fulvum*-inoculated tomato leaves with chitinase and 1,3-ß-glucanase RNA probes*

For *in situ* hybridization, antisense ^{35}S-labelled RNA probes were used. These probes were obtained from acidic (class II) and basic (class I) chitinase and 1,3-ß-glucanase cDNA clones that had previously been isolated from a cDNA library of *C. fulvum*-infected tomato plants (Danhash *et al.*, 1993; Van Kan *et al.*, 1992). *In situ* hybridization was performed on sections of tomato leaves obtained 3, 5 and 10 days post inoculation with *C. fulvum*. Expression of genes encoding acidic, apoplastic chitinase and 1,3-ß-glucanase was substantial around vascular tissue and near the epidermis of *C. fulvum*-infected tomato leaves of a compatible interaction at 10 days post inoculation (Fig. 1, A-D). In the incompatible interaction a similar cell type-specific expression of acidic chitinase and 1,3-ß-glucanase genes was observed as early as 5 days post inoculation (results not shown), but maximum level of transcript accumulation was lower than in the compatible interaction at 10 days post inoculation. The other stages of infection studied, revealed less or no accumulation of acidic chitinase and 1,3-ß-glucanase gene transcripts (results not shown). For basic chitinase and 1,3-ß-glucanase the accumulation of gene transcripts, as shown for the incompatible interaction at 5 days post inoculation (Fig. 1, E-H), was less localized when compared to accumulation of acidic chitinase and 1,3-ß-glucanase gene transcripts (Fig 1, A-D). Highest transcript accumulation for basic chitinase and 1,3-ß-glucanase was observed at 5 days post inoculation for both the compatible and the incompatible interaction (results not shown). However, for the basic 1,3-ß-glucanase (Fig. 1, E and F) the amount of transcript detected was not as high as for the basic chitinase (Fig. 1, G and H).

Control *in situ* hybridizations were performed by hybridizing sections from non-inoculated tomato leaves with antisense RNA probes from acidic and basic chitinase and 1,3-ß-glucanase and by hybridizing sections from infected tomato leaves with sense RNA probes. No specific labelling was observed in these control experiments as shown in Fig. 2.

PR protein gene expression induced by race-specific elicitors

Since race-specific elicitors are the only factors responsible for the induction of successful defence responses in tomato against *C. fulvum*, purified elicitors of *C. fulvum* were injected in leaves of different tomato genotypes to analyse whether they could specifically induce accumulation of acidic and basic chitinase and 1,3-ß-glucanase gene transcripts. Northern blot analyses revealed a higher accumulation of acidic extracellular chitinase transcripts in AVR4-injected Cf4 plants and AVR9-injected Cf9 plants compared to AVR4- and AVR9-injected Moneymaker (Cf0) plants (Fig. 3, A and B, panels chit A). The increase in acidic chitinase transcript level was clearly visible around 4 h post injection and reached a maximum around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16 to 24 h post injection for both AVR4-injected Cf4 plants (Fig. 3, A) and AVR9-injected Cf9 plants (Fig. 3, B). In tomato cultivar Moneymaker (Cf0), acidic chitinase transcripts did not accumulate after injection of the AVR9 elicitor (Fig. 3, B, panel chit A), however, some accumulation occurred upon injection of the AVR4 elicitor (Fig. 3, A, panel chit A). Increases in acidic 1,3-ß-glucanase transcript levels were observed in both the AVR4-injected Cf4 (Fig. 3, A, panel glu A) and AVR9-injected Cf9 tomato genotypes (Fig. 3, B, panel glu A). However, the acidic 1,3-ß-glucanase gene expression appears to be induced later upon injection of AVR9 in the Cf9 genotype, than expression of the acidic chitinase gene. The basic chitinase and 1,3-ß-glucanase gene transcripts accumulated in all elicitor injected combinations (Fig. 3, panels chit B and glu B), irrespective whether the plants contained the corresponding resistance gene or not. Water injected plants also revealed a low level of transcript accumulation, predominantely of basic chitinase and basic 1,3-ß-glucanase genes, as found 1, 8 and 24 h post injection (results not shown). Especially the basic PR protein gene transcription seems to be induced easily as observed at 1 h post injection in both Moneymaker and Cf9 tomato plants (Fig. 3, A and B, lanes 1), however, this early expression appeared to be transient.

Discussion

Previous expression studies that were based on northern blot analyses, revealed no differences in accumulation pattern of basic, intracellular chitinase and 1,3-ß-glucanase mRNAs, between compatible and incompatible *C. fulvum*-tomato interactions (Danhash *et al.*, 1993; Van Kan *et al.*, 1992). It was suggested that expression of basic, intracellular chitinase and 1,3-ß-glucanase genes might occur more localized in incompatible *C. fulvum*-tomato interactions, and thereby could cause a high accumulation of basic chitinase and basic

Fig. 1. Localization of acidic and basic chitinase and 1,3-ß-glucanase gene transcripts in transverse sections of *C. fulvum*-inoculated tomato leaf tissue. A, C, E, G, bright field micrographs; B, D, F, H, epipolarization micrographs. Hybridization is visible as clear white spots in the epipolarization micrographs. A, B; Transverse leaf section of a compatible *C. fulvum*-tomato interaction (race 5-Cf5) obtained 10 days post inoculation and hybridized with an acidic 1,3-ß-glucanase (glu A) antisense RNA probe (class II). Fungal hyphae (arrows) are abundantly present in the intercellular space between spongy parenchyma (S) and near palisade parenchyma (P) of the tomato leaf. Accumulation of acidic 1,3-ß-glucanase gene transcripts can be observed in the upper (U) and lower epidermis (L) as well as in the mesophyll near vascular tissue (V). C, D; Transverse section of a similar interaction as shown in A, B, hybridized with an acidic chitinase (chit A) antisense RNA probe (class II). Strong transcript accumulation is observed near vascular (V) tissue and in mesophyll adjacent to the lower epidermis (L). Palisade cells in close contact with fungal hyphae (arrows) show no increased hybridization with the acidic chitinase probe. E, F; Transverse leaf section of an incompatible *C. fulvum*-tomato interaction (race 5-Cf4), obtained 5 days post inoculation and hybridized with a basic 1,3-ß-glucanase (glu B) antisense RNA probe (class I). Little labelling is found in all tissues. Fungal hyphae were not detectable at this stage of infection. G, H; Transverse leaf section of a interaction similar as shown in E, F, hybridized with basic chitinase (chit B) antisense RNA probe (class I). Labelling is higher than observed with the basic 1,3-ß-glucanase. Some induction is found near vascular tissue (V) but not in all cases. Bar = 50 μm

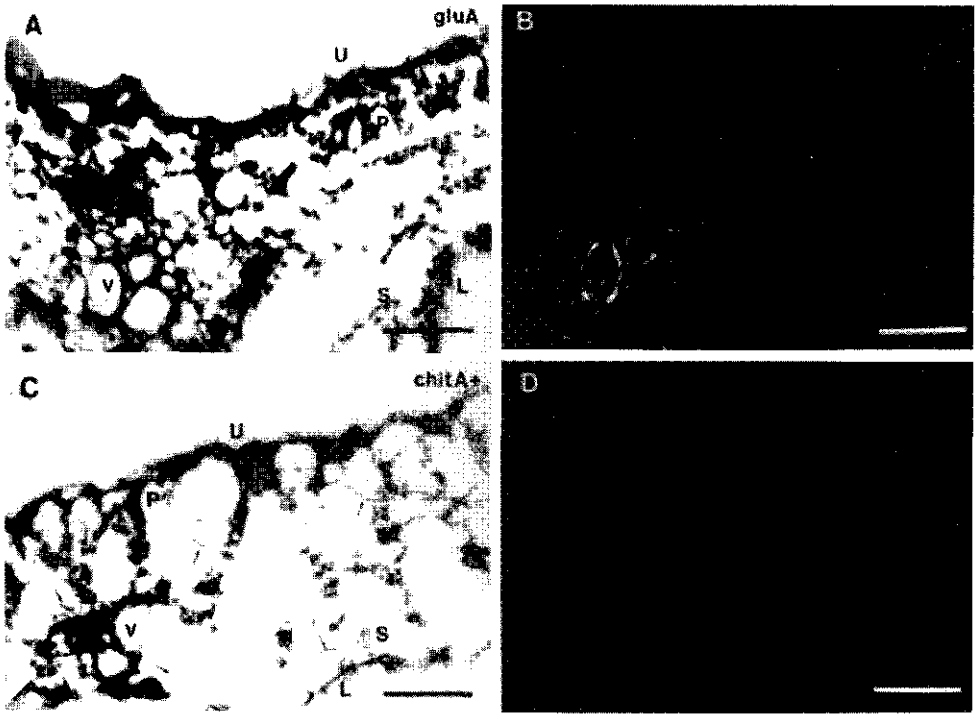


Fig. 2. Control *in situ* hybridizations in transverse sections of tomato leaves. A, C, brightfield micrographs; B, D, epipolarization micrographs; A, B; Transverse section of non-inoculated tomato leaf hybridized with an acidic glucanase (glu A) antisense RNA probe (class II). Very little hybridization is observed in healthy tomato leaf tissue. Indicated are vascular tissue (V), upper (U) and lower (L) epidermis, spongy parenchyma (S) and palisade parenchyma (P). C, D; Transverse leaf section of a compatible *C. fulvum*-tomato interaction (race 5-Cf5) obtained 10 days post inoculation and hybridized with an acidic chitinase (chit A(+)) sense RNA probe. Abundant growth of fungal hyphae (arrows) in the intercellular spaces is visible. There is very little hybridization in the tissue including vascular tissue (V), the upper (U) and lower (L) epidermis, the spongy parenchyma (S) and the palisade parenchyma (P). Bar = 50 μm

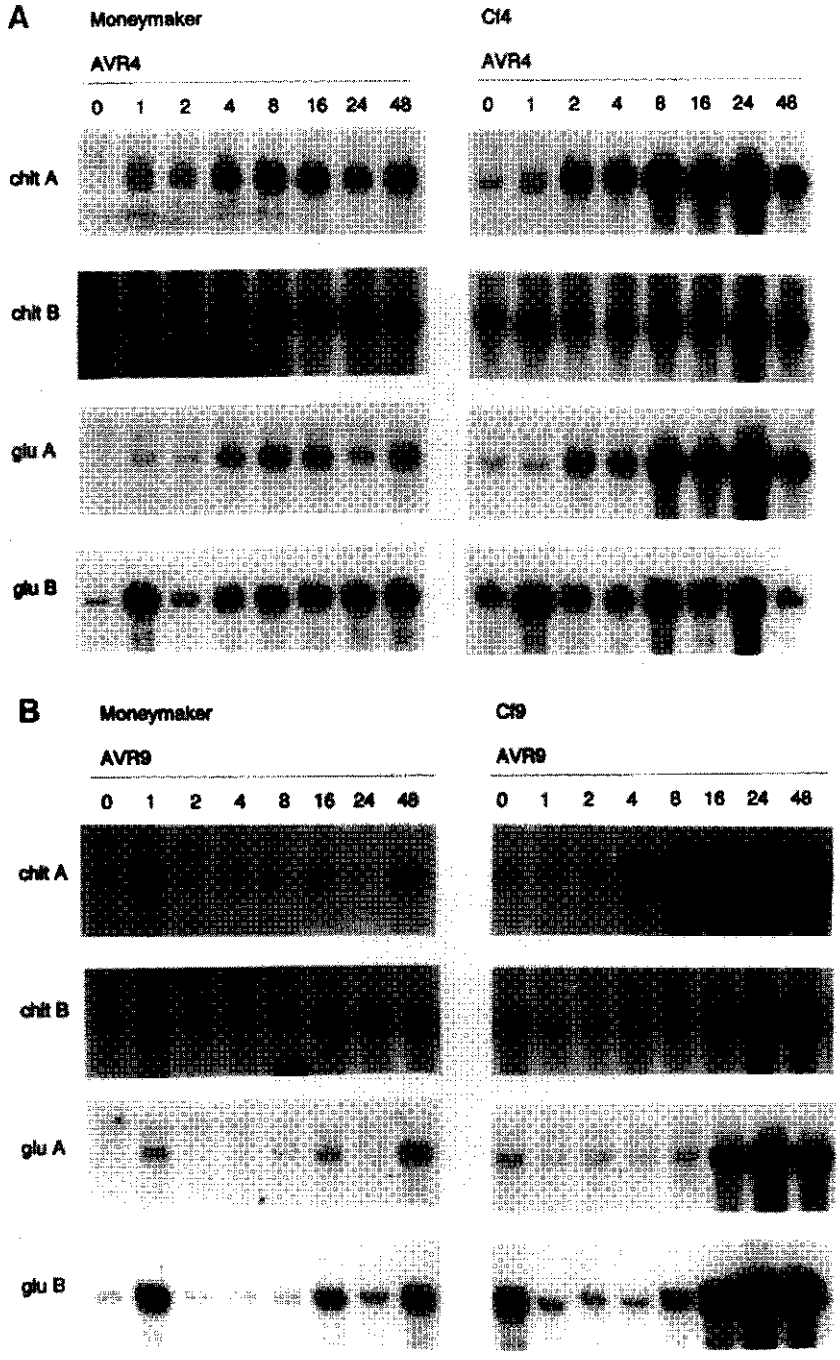
1,3-ß-glucanase in the vacuoles of guard cells and mesophyll cells near penetration sites. In this way localized accumulation might contribute to active defence against the invading pathogen during HR in incompatible interactions. However, we found transcript accumulation for the basic chitinase and 1,3-ß-glucanase at similar sites in compatible and incompatible interactions, while no local accumulation was observed at penetration sites in incompatible interactions. For acidic chitinases and 1,3-ß-glucanases, northern analyses had indicated that gene transcript accumulation occurred earlier in incompatible interactions than in compatible ones (Danhash *et al.*, 1993: Van Kan *et al.*, 1992). According to our results spatial distribution of acidic chitinase and 1,3-ß-glucanase gene transcript accumulation, as observed by *in situ* hybridization, appeared to be similar in compatible and incompatible interactions,

and only temporal differences were found. The hybridization found for the acidic chitinase and 1,3-ß-glucanase was highest in the compatible interaction at 10 days post inoculation.

Evidence for a role of chitinase and 1,3-ß-glucanase in resistance of tomato against *C. fulvum* is not clear. No differences in the basic chitinase and basic 1,3-ß-glucanase gene expression between compatible and incompatible interactions were found by northern analyses. Furthermore, an increased localized induction of intracellular basic chitinase and 1,3-ß-glucanase around the penetrating hyphae in the incompatible interaction was not found in the present study. Therefore, an important role for basic PR proteins in resistance of tomato against *C. fulvum* is not likely, unless they are released in the extracellular space of the infected tomato leaf upon HR in incompatible interactions only. For gene expression of acidic chitinase and 1,3-ß-glucanase, early induction in the incompatible *C. fulvum*-tomato interaction was observed when compared to the compatible interaction, but no spatial differences could be detected. Thus, in incompatible interactions the fungus possibly undergoes deleterious effects of these extracellular hydrolytic enzymes earlier than in compatible interactions. Whether this accounts for the successful active defence in incompatible interactions is not clear yet.

Expression studies on PR protein genes reported by other research groups revealed spatial distribution patterns of expression similar as those reported in this paper. Accumulation of chitinase and phenylalanine ammonia-lyase (PAL) transcripts near vascular tissue and epidermal layers was found in young, healthy leaves of potato and parsley (Kombrink *et al.*, 1993), while induced accumulation of these transcripts was found around necrotic lesions caused by different *Phytophthora* species (Kombrink *et al.*, 1993; Schröder *et al.*, 1992). In potato leaves infected by *Phytophthora infestans*, chitinase and 1,3-ß-glucanase protein and mRNA distribution patterns were nearly identical in compatible and incompatible interactions (Schröder *et al.*, 1992). Samac and Shah (1991) studied chitinase gene expression in transgenic tomato containing an *Arabidopsis thaliana* acidic chitinase (class III) promoter fused to the *Gus* reporter gene. In healthy plants they found GUS activity near leaf vascular tissue, guard cells and hydathodes. This spatial distribution pattern is similar to our observations for the extracellular, acidic chitinase (class II) in *C. fulvum*-infected tomato plants. Different classes of PR proteins can give different expression patterns, as was observed in our studies when comparing the class I chitinase and 1,3-ß-glucanase with the class II proteins. These differences should be considered when discussing the relevance of the spatial distribution of PR gene expression in plant-pathogen interactions. Additional information should be available on different classes of PR proteins in the plant-fungus interaction under study before any conclusion on their role in defence can be drawn.

The acidic chitinase and 1,3-ß-glucanase mRNA accumulation occurs in an elicitor-dependent manner in genotypes with the matching genes for resistance, as observed after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AVR4
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B
Moneymaker
Cf9
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0 1 2 4 8 16 24 48
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glu B

injection of Cf4 and Cf9 plants with purified AVR4 and AVR9 race-specific elicitors, respectively. This differential elicitor-dependent expression pattern is in agreement with the observed differences in mRNA accumulation patterns observed between compatible and incompatible *C. fulvum*-tomato interactions. Significant accumulation of acidic chitinase and acidic 1,3-ß-glucanase gene transcripts (Danhash *et al.*, 1993; Van Kan *et al.*, 1992) and the encoded proteins occurred in the incompatible interaction as early as 4 days after inoculation (Joosten and De Wit, 1989). At that stage of infection the fungus is penetrating the tomato leaf through stomata and at the same time expression of the avirulence gene *avr*9 is induced, as shown by GUS expression studies (Van den Ackerveken *et al.*, 1994). Only in incompatible interactions the elicitor is recognized and as part of the defence response acidic chitinase and 1,3-ß-glucanase gene expression is induced as early as 2 to 4 h post perception. The late accumulation of acidic chitinase and 1,3-ß-glucanase in compatible interactions is probably due to non-specific stress resulting from extensive colonization of the extracellular spaces of the tomato leaf. Moreover, deprivation of nutrients and non-specific elicitors might cause induction of acidic chitinase and 1,3-ß-glucanase gene expression at that stage of infection in compatible interactions. Furthermore, preliminary results, observed by *in situ* hybridization studies, indicated that cell type-specific gene expression is similar in elicitor treated and *C. fulvum*-infected tomato (unpublished results). For basic chitinase and basic 1,3-ß-glucanase, no race-specific elicitor-mediated induced gene expression was observed with the AVR4 elicitor, while the AVR9 elicitor only showed a low level of induction. Early induction of the latter genes in infected tomato leaves is possibly due to recognition of non-specific elicitors in both resistant and susceptible tomato genotypes. A possible race-specific elicitor-mediated induction of basic chitinase and 1,3-ß-glucanase in the *C. fulvum*-tomato interaction is masked by this non-specific early induction. Furthermore, basic chitinase and 1,3-ß-glucanase gene expression was observed in elicitor and water injected tomato leaves as early as 1 h post injection, indicating early transient induction of gene expression upon wounding caused by the injection procedure. It has been shown for several plants that basic chitinase and 1,3-ß-glucanase gene expression could be induced upon wounding which transiently releases ethylene known to induce predominantly basic PR proteins (Beerhues and Kombrink, 1994; Brederode *et al.*, 1991; Mauch *et al.*, 1988a).

In addition to a role in the resistance gene-mediated defence response, PR protein

Fig. 3. Northern blot analysis of total RNA (15 μg) isolated from AVR4 (A) and AVR9 (B) injected tomato leaves. Blots were hybridized with acidic and basic chitinase and 1,3-ß-glucanase DNA probes. RNA was isolated from AVR4-injected (100 μl leaf^{-1}, 10 ng AVR4 μl^{-1}) Moneymaker (Cf0) and Cf4 tomato leaves (A, left and right panels, respectively) and AVR9-injected (100 μl leaf^{-1}, 2 ng AVR9 μl^{-1}) Moneymaker (Cf0) and Cf9 leaves (B, left and right panels, respectively). Injected tissue was frozen in liquid nitrogen at 0 (control), 1, 2, 4, 8, 16, 24, and 48 h post injection. Hybridization was performed with ^{32}P-labelled random primed DNA probes from acidic chitinase (chit A), basic chitinase (chit B), acidic 1,3-ß-glucanase (glu A) and basic 1,3-ß-glucanase (glu B).

accumulation is thought to play a role in acquired resistance as well. Only limited evidence has been obtained indicating the existence of local (LAR) and systemic acquired resistance (SAR) in tomato (Christ and Mösinger, 1989; Heller and Gessler, 1986). Inoculation of tomato genotype Cf4 with an avirulent race of *C. fulvum* (race 5) was shown to induce only local and no systemic resistance against *Phytophthora infestans* (Christ and Mösinger, 1989). Local chlorosis or necrosis induced by avirulent race 5 of *C. fulvum* on tomato genotype Cf4 is probably not sufficient to induce systemic resistance. It is possible that combinations of other avirulent races of *C. fulvum* with resistant genotypes of tomato known to develop a stronger resistance response (Hammond-Kosack and Jones, 1994) could induce SAR in tomato. Currently, we are investigating induction of PR gene expression in tomato triggered by compounds which have reported to be involved in SAR in other plant-pathogen interactions. The involvement of different chitinase and 1,3-ß-glucanase isoforms with acquired resistance in tomato, combined with the induced PR gene expression either locally or systemically, as triggered by specific and non-specific elicitors, can provide substantial information on the phenomenon of SAR in tomato.

Materials and methods

Plants and fungi

Tomato genotypes Cf4, Cf5, Cf9 and Moneymaker (Cf0) were grown as described before (De Wit and Flach, 1979). For inoculations, conidia were obtained from *Cladosporium fulvum* (syn. *Fulvia fulva* (Cooke) Cif.) race 5 grown on potato dextrose agar for 7 to 10 days (De Wit and Flach, 1979). Four-week-old tomato plants were inoculated 3 times by spraying with a suspension of $2 \cdot 10^7$ conidia ml^{-1} and plants were incubated as described before (De Wit and Flach, 1979).

In situ *hybridization*

In situ hybridizations were performed essentially as described by Cox and Goldberg (1988). Leaf discs (2-3 mm^2) were obtained from inoculated tomato plants at 3, 5 and 10 days post inoculation. Fixation of the leaf discs was performed in 0.1 M sodium phosphate buffer (pH 7.0), 4 % formaldehyde, 0.25 % glutaraldehyde and 0.1 % Triton X-100. Leaf samples were incubated under reduced pressure at room temperature for 2 h, followed by dehydration in a graded series of ethanol. Subsequently, ethanol was replaced by xylol and leaf discs were embedded in paraplast plus. Sections of 7 μm were obtained with a Leitz rotary microtome using Feather S35 microtome blades and mounted on poly-L-lysine-coated microscope slides. Prior to hybridization, paraplast was removed with xylol and tissue was rehydrated in a graded series of ethanol. Sections were pretreated with proteinase K (1 $\mu g\ ml^{-1}$) in 100 mM Tris/HCl (pH 7.5) and 50 mM EDTA, for 30 min at 37 °C followed by 0.25 % acetic acid

anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature. The sections were dehydrated again in a graded series of ethanol and air-dried. Sense and antisense RNA probes were obtained from DNA fragments of approximately 150-200 bp cDNA clones of acidic, class II chitinase (Chi3, nucleotides 388-544; Danhash *et al.*, 1993), basic, class I chitinase (Chi9, nucleotides 0-190; Danhash *et al.*, 1993), acidic, class II 1,3-ß-glucanase (Glu A, nucleotides 618-768; Van Kan *et al.*, 1992) and basic, class I 1,3-ß-glucanase (Glu B, nucleotides 428-610; Van Kan *et al.*, 1992), which were subcloned in pGEM-3Zf(+) (Promega). Antisense RNA probes obtained from the cDNA subclones, were labelled with ^{35}S-UTP (> 1000 Ci/mmol, Amersham, UK) using the Riboprobe Gemini Transcription system (Promega). After hybridization in a solution consisting of 50 % (v/v) formamide, 300 mM NaCl, 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 x Denhardt's, 10 % (w/v) dextran sulphate, 60 mM DTT, 150 μg ml^{-1} yeast tRNA and 500 μg ml^{-1} poly(A) at 42 °C for 16 h, the hybridization mix was removed by stirring in 4 x SSC, 5 mM DTT, for 5 min 3 times. Subsequently, sections were treated with 50 μg ml^{-1} RNaseA in 0.5 M NaCl, 10 mM Tris/HCl (pH 7.5) and 5 mM EDTA, for 30 min at 37 °C. RNaseA was removed by 3 wash steps of 30 min with the same buffer, containing 5 mM DTT, at 37 °C. A final wash was performed with 2 x SSC for 30 min at room temperature, followed by dehydration in a graded ethanol series containing 0.3 M ammonium acetate, and drying under vacuum. Autoradiography was performed by coating the sections with LM-1 emulsion (Amersham, UK), diluted 1:1 in 0.6 M ammonium acetate, and incubating for 2-6 weeks at 4 °C. Slides were developed with Kodak D19 developer for 5 min and fixed in Kodak Fix. Sections were stained with 0.05 % toluidine blue for 3 min and after dehydration mounted in DePeX. Sections were examined with a Zeiss Axioscope microscope equipped with epipolarization filters (IGS) and photomicrographs were obtained with a Zeiss MC-100 camera unit (Zeiss, Germany).

Injections of race-specific elicitors in tomato leaves

Diluted preparations of purified race-specific elicitors AVR4 (Joosten *et al.*, 1994) and AVR9 (Van den Ackerveken *et al.*, 1993) were injected in leaves on tomato plants at concentrations which caused visible necrosis in the injected area at 48 h post injection (AVR4, 100 μl per leaf at 10 ng μl^{-1}; AVR9, 100 μl per leaf at 2 ng μl^{-1}).

Isolation of RNA and northern analysis

Total RNA was isolated from 1 g of frozen tomato leaf tissue using the protocol from the Extract-A-Plant RNA isolation kit (Clontech, Palo Alto, CA). Fifteen μg of total RNA was separated by 1.5 % denaturing formaldehyde agarose gel electrophoresis and subsequently blotted on Hybond-N+ membrane (Amersham, UK). ^{32}P-labelled DNA probes were obtained with the Random Primers Labeling System (BRL Life technologies Inc., USA) and

hybridization was performed in 1 mM EDTA, 0.5 M phosphate buffer (pH 7.2) and 7 % (w/v) SDS at 65 °C for 16 h. Subsequently, the blot was washed twice with 2 x SSC, 0.5 % (w/v) SDS at 65 °C for 30 min. Autoradiography was performed at - 80 °C on Kodak XAR-5 film.

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chapter 7

Expression and localization of two *in planta* induced extracellular proteins of the fungal tomato pathogen *Cladosporium fulvum*

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Abstract. Expression of two *in planta* induced genes of the biotrophic fungal tomato pathogen *Cladosporium fulvum* and accumulation of their products, extracellular proteins (ECPs), were studied in time and space during pathogenesis. Immunogold localization revealed that proteins ECP1 and ECP2 accumulated abundantly in extracellular material in the vicinity of fungal and host cell walls. Expression of the genes encoding ECP1 and ECP2 was studied in transformants carrying the reporter gene *Gus* fused to promoter sequences of the *ecp* genes. In germinated conidia on the leaf surface no expression of *ecp*1 and only low expression of *ecp*2 could be detected. Expression of both *ecp*1 and *ecp*2 genes was strongly induced during colonization of the intercellular space between tomato mesophyll cells. The highest expression was observed in hyphae growing near the vascular tissue. Expression levels were low in newly formed conidia on leaves. Possible functions of ECP1 and ECP2 for *C. fulvum* during pathogenesis are discussed.

Introduction

Most plant pathogenic fungi are specialized and capable of infecting only one or a few plant species. These fungi are able to circumvent the induction of a defence response and will depend upon pathogenicity factors which may be involved in the formation of infection structures such as appressoria (Talbot *et al.*, 1993) or haustoria; degradation of host cell walls (Maiti and Kolattukudy 1979; Dickman *et al.*, 1989); or detoxification of phytoalexins (Schäfer *et al.*, 1989) or plant toxins (Osbourn *et al.*, 1991). Two putative pathogenicity factors that have been studied in detail are cutinase and pisatin demethylase of the pea pathogen *Nectria haematococca* (Maiti and Kolattukudy 1979; Dickman *et al.*, 1989 Schäfer *et al.*, 1989). Recent data indicate that the genes encoding cutinase and pisatin demethylase are not strictly required for pathogenicity, as disruption of these genes did not result in a detectable decrease of pathogenicity (Stahl and Schäfer 1992; VanEtten *et al.*, 1993). These results indicate that a directed functional approach to estimate the importance of single genes in the pathogenicity of fungal pathogens is often not successful. An unbiased approach was followed by Talbot *et al.* (1993) studying the pathogenicity of the rice blast fungus *Magnaporthe grisea*; using differential screening in a search for genes induced *in planta*, they cloned the *MPG1* gene. Disruption of this gene resulted in reduced pathogenicity of *M. grisea* on rice, possibly as a result of impaired appressorium formation.

We are investigating the interaction between the plant pathogenic fungus *Cladosporium fulvum* Cooke (syn. *Fulvia fulva* (Cooke) Cif.) and its only host, tomato (*Lycopersicon esculentum* Mill). *C. fulvum* is a biotrophic pathogen which grows in the intercellular space of the leaf without penetrating the mesophyll cells (Lazarovits and Higgins 1976a,b; De Wit 1977; De Wit and Flach 1979). Extensive studies of molecular aspects of the gene-for-gene relationship between *C. fulvum* and tomato have resulted in cloning and

characterization of two fungal avirulence genes (Van Kan *et al.*, 1991; Van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). The corresponding resistance genes in tomato have not yet been cloned. Several defence-related responses are activated after initial contact between avirulent races of *C. fulvum* and resistant tomato plants, one of which is the accumulation of pathogenesis-related proteins. Several of the pathogenesis-related proteins from tomato have been purified to homogeneity (Joosten *et al.*, 1989; Joosten *et al.*, 1990a) and genes encoding these proteins have been cloned (Danhash *et al.*, 1993; Linthorst *et al* 1991; Van Kan *et al.*, 1992). The role of these pathogenesis-related proteins in the defence of tomato against *C. fulvum* is still unclear.

Pathogenicity factors of *C. fulvum* have been studied in less detail. In search of these factors we examined the proteins present in the intercellular space of *C. fulvum*-infected tomato leaves. Several low molecular weight proteins (20 kD or less), of supposed fungal origin, have been found in apoplastic fluid of *C. fulvum*-infected leaves of susceptible tomato plants, but not in culture filtrate of *C. fulvum* grown *in vitro* (De Wit *et al.*, 1989). Three extracellular proteins (ECPs) have been purified to homogeneity, polyclonal antibodies have been raised (Joosten and De Wit 1988; M.H.A.J. Joosten, unpublished) and the genes encoding ECP1 and ECP2 have been cloned from race 5 of *C. fulvum* (Van den Ackerveken

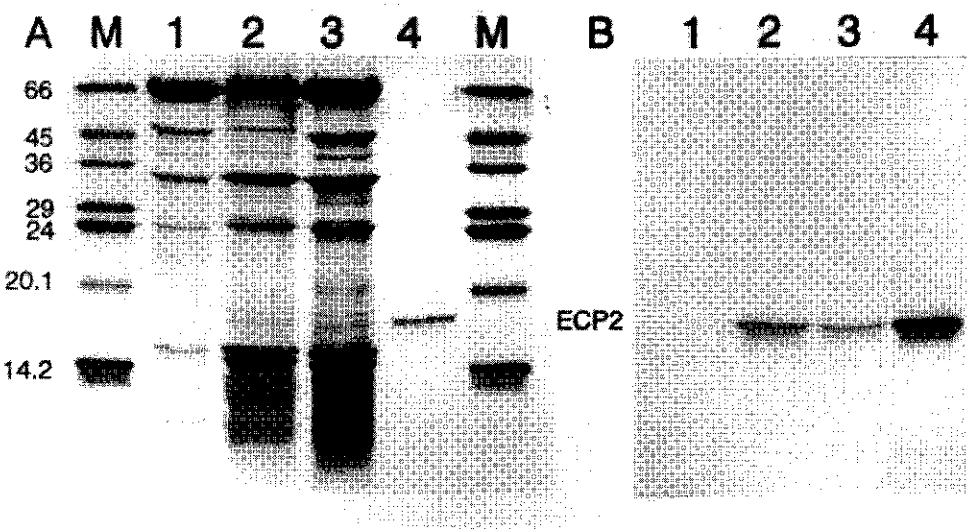


Fig. 1. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 50 µl samples of apoplastic fluid obtained from tomato genotype Cf4 in an incompatible interaction with *Cladosporium fulvum* race 5 (lane 1), from genotype Cf4 in a compatible interactions with race 4 (lane 2), and from genotype Cf5 in a compatible interaction with race 5 (lane 3), at 14 days after inoculation, and of purified ECP2 (lane 4). The two outer lanes (M) contain molecular weight markers (kD). B, Immunoblot of a gel similar to the one in A, incubated with antibodies raised against ECP2. The antibodies have a high affinity to the purified protein (lane 4) and detect ECP2 only in apoplastic fluid from compatible interactions (lanes 2 and 3), but not in that from an incompatible interaction.

et al., 1993). The genes, *ecp*1 and *ecp*2, appear to be highly expressed *in planta*, whereas their expression is low or undetectable in the fungus grown *in vitro* (Van den Ackerveken *et al.*, 1993).

Here we describe the purification of ECP2 and present data on the *in situ* localization of both ECP1 and ECP2 in *C. fulvum*-infected tomato leaves. The expression of the *ecp*1 and *ecp*2 genes in time and space during pathogenesis was studied using transformants of *C. fulvum* carrying *ecp* promoter-*Gus* fusions. Possible functions of ECP1 and ECP2 for *C. fulvum* during pathogenesis on tomato are discussed.

Results

Purification of the ECP proteins

The purification of the protein ECP1 was described previously (Joosten and De Wit 1988).

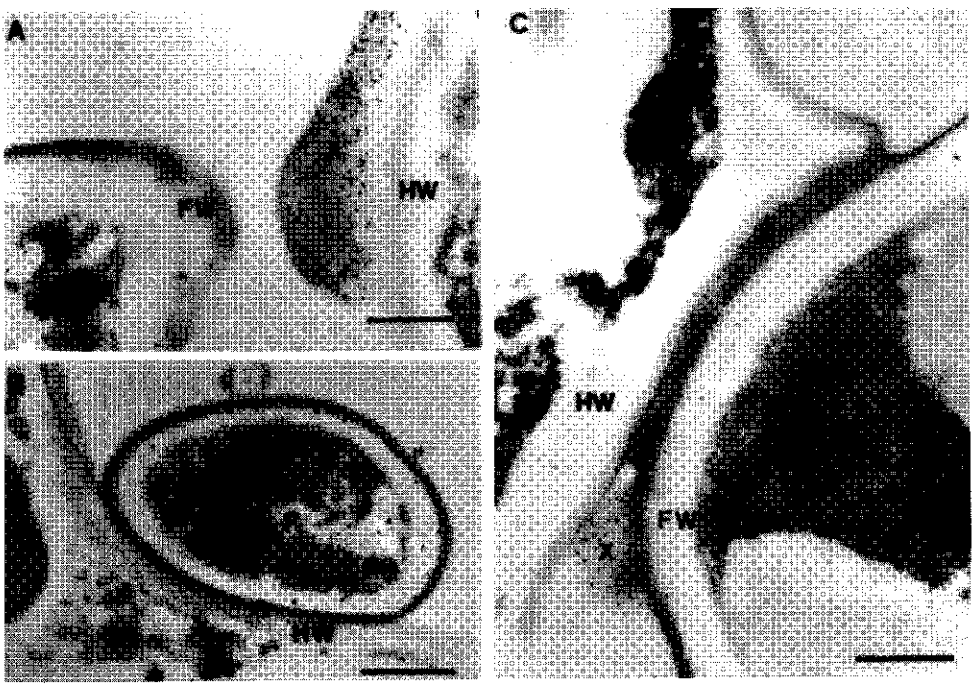


Fig. 2. Immunocytological localization of ECP1 and ECP2 in tomato leaves inoculated with *Cladosporium fulvum*. Immunogold labelling of ECP1 (A) and ECP2 (B and C) was performed on ultrathin sections of leaf material from tomato genotype Cf5 in a compatible interaction between *C. fulvum* race 5, at 12 days after inoculation. Gold particles are visible in fungal hyphae (F) and in extracellular material (X) between the fungal cell wall (FW) and the wall of the mesophyll cells (HW). In control experiments, performed on uninoculated tomato leaves with the specific antibodies and on inoculated leaves with preimmune serum, no labelling was observed (results not shown). Bar = 0.5 μm.

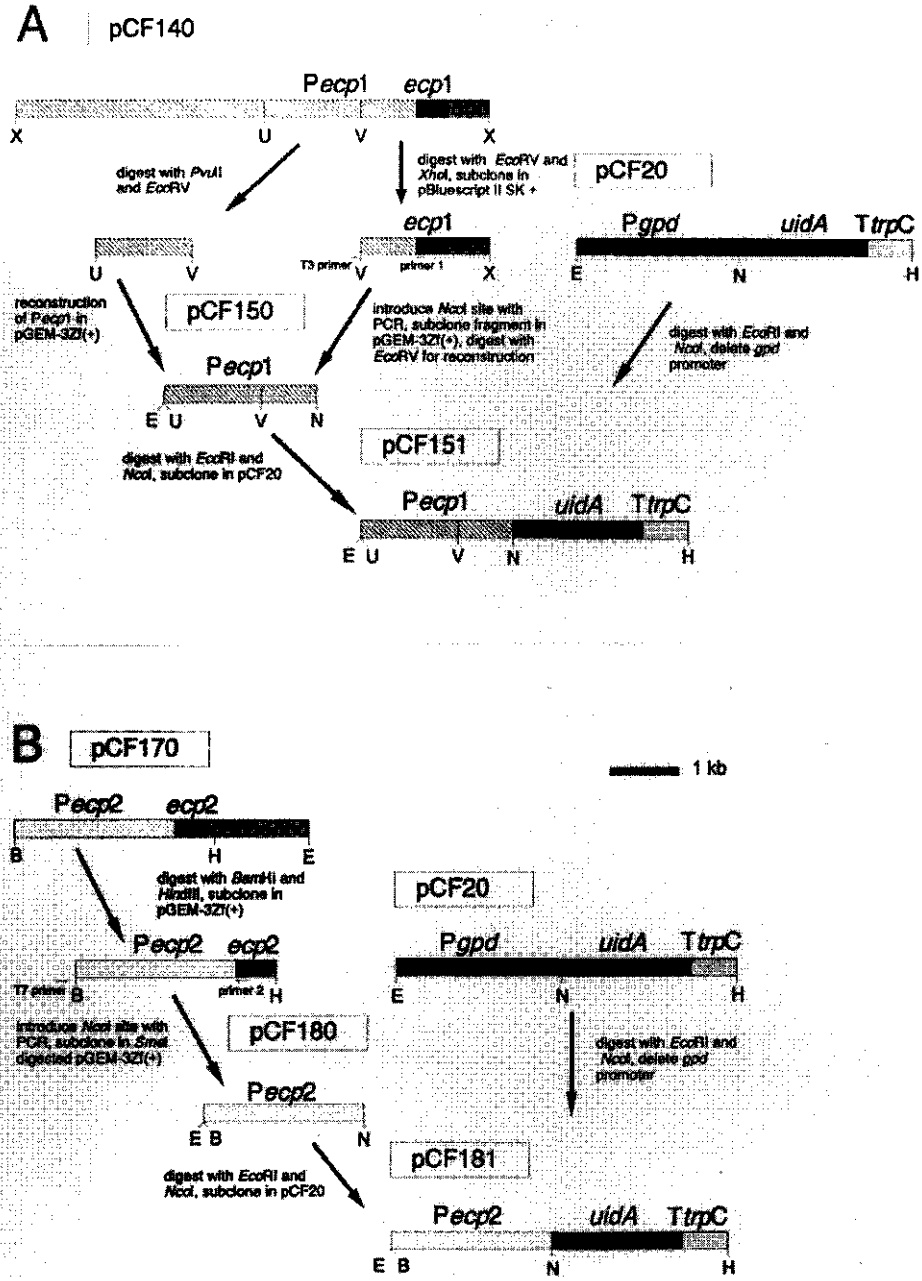
The protein ECP2 was purified to homogeneity from apoplastic fluid originating from a compatible interaction between tomato genotype Cf5 and race 5 of *C. fulvum* by gel filtration followed by anion exchange chromatography (Fig. 1, A, lane 4). From the first anion exchange run, following fractionation on Sephadex G-50, two peaks containing ECP2 were obtained: one eluting around 110 mM NaCl and the other eluting around 160 mM NaCl. Rechromatography of each fraction containing ECP2 resulted again in the same two peaks. After analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a single protein band of 17 kD was present in both peaks while in native, high pH PAGE the ECP2 protein resolved into two separate bands (results not shown). The protein probably exists in two conformations which reversibly change under non-denaturing conditions.

Western blot analysis, using polyclonal antibodies raised against the purified ECP2, revealed that the protein was present in apoplastic fluids obtained from a compatible interaction between *C. fulvum* and tomato (Fig. 1, B, lanes 2 and 3). ECP2 was not detected in culture filtrate of *C. fulvum* grown *in vitro* (results not shown), in apoplastic fluids obtained from an incompatible interaction between *C. fulvum* and tomato (Fig. 1, B, lane 1), nor in healthy tomato plants (results not shown).

Immunolocalization of ECP1 and ECP2 in infected tomato leaves

Immunolocalization experiments were performed on *C. fulvum* inoculated tomato leaves in compatible and incompatible interactions, obtained at 7 and 12 days post inoculation. Polyclonal antibodies used were raised against the purified ECP1 and ECP2 proteins. Accumulation of ECP1 and ECP2 in the apoplast of *C. fulvum*-infected tomato leaves was observed at 12 days post inoculation. Both proteins accumulated at similar locations in *C. fulvum*-infected tomato leaves in a compatible interaction at 12 days post inoculation, primarily in extracellular material present in the vicinity of fungal and host cell walls (Fig. 2, A and C). The proteins were detected in low quantities in the cytoplasm of growing hyphae (Fig. 2, A-C), and in the extracellular matrix between leaf mesophyll cells (results not shown). However, ECP1 and ECP2 were never detected inside tomato mesophyll cells (Fig.

Fig. 3. Construction of transformants of *Cladosporium fulvum* containing the coding region (*uidA*) of the reporter gene *Gus* fused to promoter sequences of the *ecp*1 and *ecp*2 genes. A, To construct the *ecp*1 promoter-*Gus* fusion, the *gpd* promoter (P*gpd*) present in pCF20 (Van den Ackerveken *et al.* 1994) was deleted by digestion with *Eco*RI and *Nco*I and replaced by a 2.2-kb *ecp*1 promoter fragment (P*ecp*1) isolated from pCF140 (Van den Ackerveken *et al.* 1993). An *Nco*I site was introduced at the ATG startcodon of the *ecp*1 gene with polymerase chain reaction (PCR); and after reconstruction of the promoter in pGEM-3ZF(+) (pCF150) this plasmid was digested with *Eco*RI and *Nco*I and the 2.2-kb P*ecp*1 region isolated and ligated to the *uidA* coding region in pCF20 (pCF151). B, A similar approach was used for the fusion of the promoter of *ecp*2 with the *Gus* coding region (*uidA*). The *gpd* promoter (P*gpd*) in pCF20 was replaced by a 2.3-kb *ecp*2 promoter fragment (P*ecp*2) from pCF170 (Van den Ackerveken *et al.* 1993). With PCR, an *Nco*I site was introduced at the ATG startcodon of the *ecp*2 gene and this PCR product was subcloned in pGEM-3Zf(+) (pCF180). The 2.3-kb P*ecp*2 fragment was isolated from pCF180 by digestion with *Eco*RI and *Nco*I and ligated to the *uidA* gene in pCF20 to obtain pCF181. B=*Bam*HI, E=*Eco*RI, H=*Hin*dIII, N=*Nco*I, U=*Pvu*II, V=*Eco*RV, and X=*Xho*I.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pCF140
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T3 primer
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primer 1
X
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N
H
reconstruction of Pecp1 in pGEM-3Zf(+)
pCF150
introduce NcoI site with PCR, subclone fragment in pGEM-3Zf(+), digest with EcoRV for reconstruction
digest with EcoRI and NcoI, delete gpd promoter
Pecp1
E U
V
N
digest with EcoRI and NcoI, subclone in pCF20
pCF151
Pecp1
uidA
TtrpC
E U
V
N
H
B
pCF170
1 kb
Pecp2
ecp2
B
H
E
digest with BamHI and HindIII, subclone in pGEM-3Zf(+)
pCF20
Pecp2
ecp2
Pgpd
uidA
TtrpC
T7 primer
B
primer 2
H
E
N
H
introduce NcoI site with PCR, subclone in SmaI digested pGEM-3Zf(+)
pCF180
digest with EcoRI and NcoI, delete gpd promoter
Pecp2
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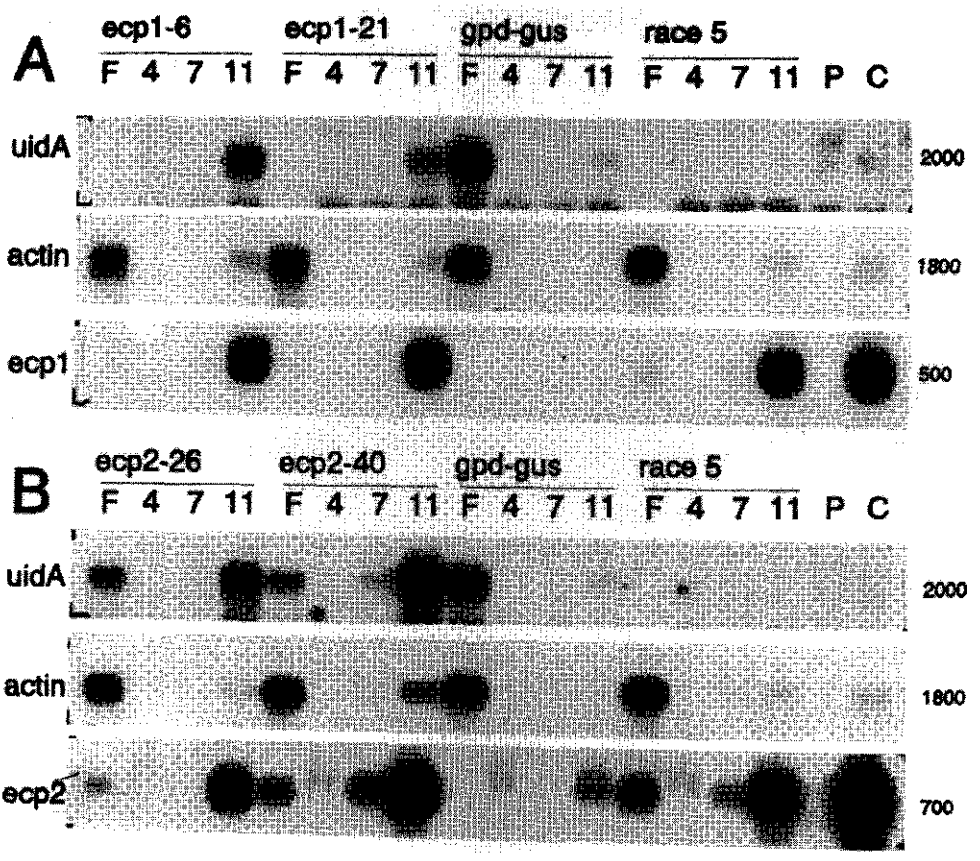


Fig. 4. Northern blot analysis of total RNA (10 μg) isolated from tomato seedlings infected by *Cladosporium fulvum* at 4, 7, and 11 days after inoculation (lanes 4, 7, and 11, respectively) and from *C. fulvum* grown in liquid shake culture (lane F in each series). Blots of the transformants of ecp1-6 and ecp1-21 of *C. fulvum*, with the P*ecp*1-*Gus* construct (A), and of the transformants ecp2-26 and ecp2-40, with the P*ecp*2-*Gus* construct (B), were hybridized with *ecp*1 and *ecp*2, respectively, and with the *uidA* and actin genes. RNA of the constitutively expressing GUS transformant (gpd-gus) and of the untransformed *C. fulvum* race 5 was blotted as well. Lane P contains RNA from leaves of uninoculated tomato plants and lane C contains RNA from leaves of infected susceptible tomato (genotype Cf5 infected by race 5), isolated at 14 days after inoculation.

2, A-C). Occasionally, ECP1 and ECP2 were detected in and near intact mycelium in incompatible interactions (results not shown).

Characterization of transformants of C. fulvum *containing the* Pecp-Gus *constructs*

The cloning of the *ecp*1 and *ecp*2 genes from *C. fulvum* has been described previously (Van den Ackerveken *et al.*, 1993). The regulation of the expression of *ecp*1 and *ecp*2 during

infection of tomato was studied with transformants of *C. fulvum* containing the coding region of the reporter gene *Gus* (*uidA*), encoding ß-glucuronidase (GUS), fused to promoter sequences of *ecp*1 or *ecp*2 (P*ecp1* or P*ecp2*, respectively). The construction of the P*ecp*1-*Gus* and P*ecp2*-*Gus* fusions is depicted in Figure 3. Perfect fusions of the *ecp* promoter fragments with *Gus* were made by introducing an *Nco*I site at the ATG startcodon of the *ecp* genes with polymerase chain reaction (PCR). This *Nco*I site was subsequently used for the fusion to the coding region of the *Gus* gene (*uidA*) in pCF20. The *ecp* promoter-*Gus* fusion constructs were introduced into *C. fulvum* race 5 by cotransformation using the vector pAN7-1 containing the hygromycin B resistance gene as a selection marker. Cotransformation was assessed by screening different hygromycin-resistant P*ecp*-*Gus* transformants for GUS activity *in vitro* (grown on potato-dextrose agar) and *in planta* (grown on susceptible tomato seedlings) (results not shown). Several transformants of *C. fulvum* were used for Southern analysis. All transformants which revealed induced GUS activity *in planta* contained at least one intact copy of the P*ecp*1-*Gus* or P*ecp2*-*Gus* fusion construct (results not shown). Four representative transformants, which revealed an increased GUS activity *in planta* and contained a low number of intact insertions of the particular P*ecp*-*Gus* construct, were analyzed by northern blotting (ecp1-6 and ecp1-21, ecp2-26 and ecp2-40). Transcript levels of *uidA*, *ecp*1 and *ecp*2 were compared after culture of the transformants *in vitro* and *in planta*. In both the P*ecp*1-*Gus* and P*ecp2*-*Gus* transformants, the level of *uidA* transcript was similar to that of endogenous *ecp*1 and *ecp*2 genes (Fig. 4, A, ecp1-6 and ecp1-21, and Fig. 4, B, ecp2-26 and ecp2-40). As controls, similar studies were performed with a transgenic race 4 of *C. fulvum* containing the *uidA* gene fused to the constitutive *gpd* promoter of *Aspergillus nidulans* (P*gpd*-*Gus*) (Fig. 4, gpd-gus) and with an untransformed race 5 of *C. fulvum* (Fig. 4, race 5). In the P*gpd*-*Gus* transformant grown *in vitro*, a high level of *uidA* transcript was observed, while in the P*ecp*1-*Gus* transformants grown under similar conditions no *uidA* transcript was detected (Fig. 4, A, lanes F), and in the P*ecp2*-*Gus* transformants only low amounts of *uidA* transcript were observed (Fig. 4, B, lanes F). The P*gpd*-*Gus* transformant, however, showed a reduced *ecp*1 and *ecp*2 expression *in planta* compared to the P*ecp*-*Gus* transformants and the untransformed *C. fulvum* race 5 (Fig. 4, lanes 4, 7 and 11). This may be due to a difference in the genetic background of the two types of transformants which relates to the reduced growth of race 4, compared to race 5 (Joosten *et al.*, 1990b). In untransformed race 5 of *C. fulvum* no *uidA* transcript could be detected while the transcript level of *ecp*1 and *ecp*2 was similar as for the P*ecp*-*Gus* transformants (Fig. 4, race 5). The constitutively expressed *C. fulvum* actin gene was used to quantify the amount of fungal RNA in each sample (Van den Ackerveken *et al.*, 1993). The actin transcript was abundantly present in the fungus grown *in vitro* (Fig. 4, lanes F). Increasing levels of actin transcript were observed *in planta* (Fig. 4, lanes 4, 7 and 11), indicating that the amount of fungal RNA in the samples increases as the infection proceeded.

Table 1. ß-Glucuronidase (GUS) activity of the P*ecp-Gus* transformants of *Cladosporium fulvum* grown *in vitro* or in susceptible tomato seedlings[a]

Growth stage	Days after inoculation	P*ecp1-Gus*	P*ecp2-Gus*
Fungus grown *in vitro* on potato-dextrose agar	...	-	+
Conidia on leaf surface	...	-	+
Germ tubes	1-2	-	+
Runner hyphae	2-4	-	+
Penetration hyphae	4-5	-	+
Thickened hyphae in mesophyll	5-10	++	++
Branched hyphae near vascular tissue	5-10	++	++
Substomatal primordia	10-14	++	++
Conidiophores	10-16	++	++
Conidia	12-16	-	+

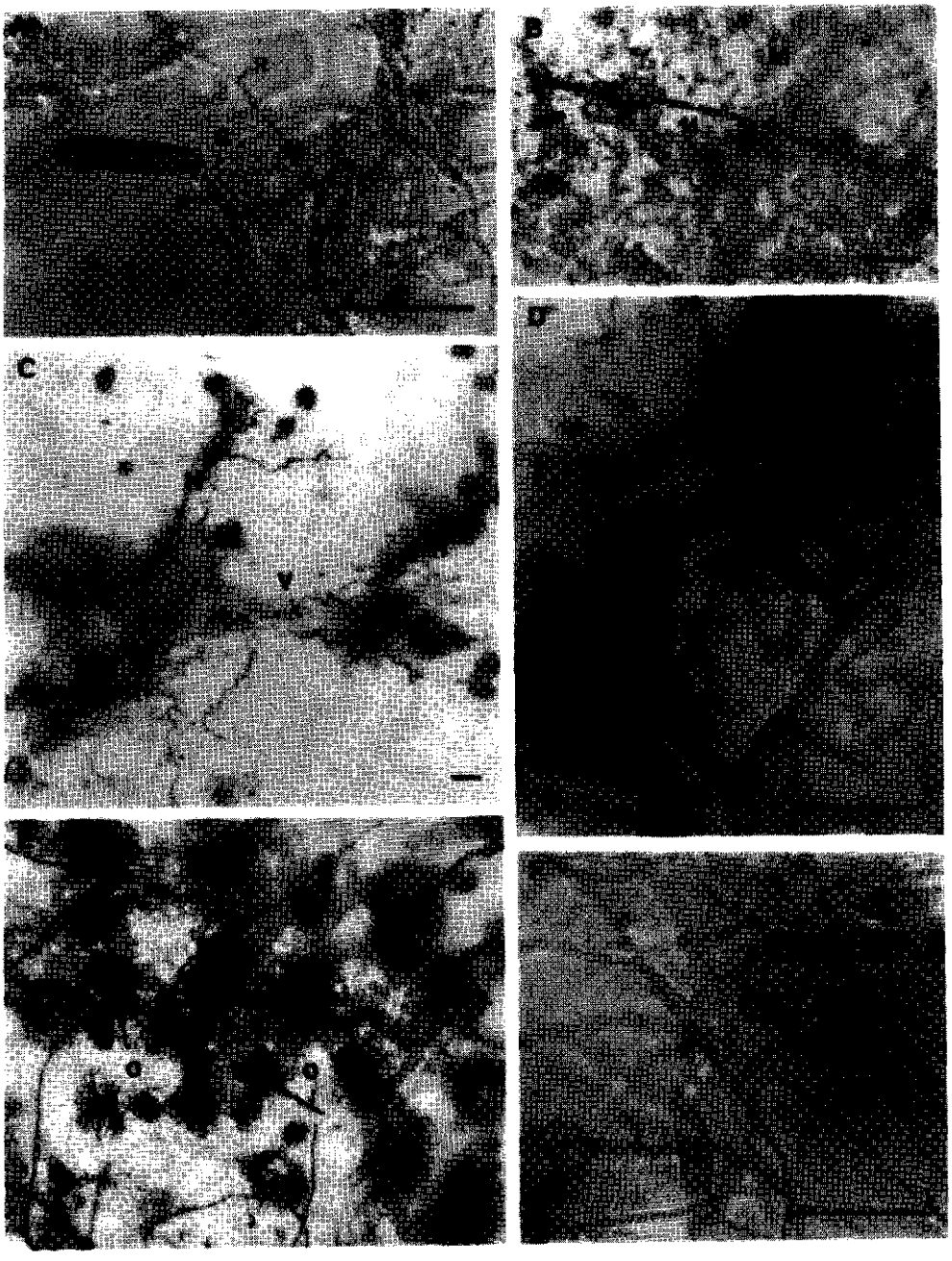
[a]GUS activity was estimated by studying three or four leaflets of infected 3-wk-old tomato seedlings, stained for GUS activity as described in Materials and Methods, at 4, 6, 10, 12, and 16 days post inoculation. -, No GUS activity observed (Fig. 5A and F). +, Low GUS activity observed in some hyphae (approximately one out of ten). ++, Consistent strong GUS activity in all hyphae (Fig. 5B-E).

The P*gpd-Gus* transformant grown *in planta* contained very low actin mRNA levels indicating that the growth of this transformant (race 4) *in planta* is much less heavy than the growth of the other transformants (race 5) of *C. fulvum*.

Expression of the ecp1 and ecp2 genes during pathogenesis

The P*ecp-Gus* transformants of *C. fulvum* were used to study the expression of the *ecp*1 and *ecp*2 genes in time and space during pathogenesis. GUS activity was estimated at different stages of infection of 3-wk-old tomato seedlings that had been inoculated with the different

Fig. 5. Histochemical localization of ß-glucuronidase (GUS) activity in susceptible tomato seedlings inoculated with the *ecp* promoter-*Gus* transformants of *Cladosporium fulvum*. Inoculated leaflets of tomato seedlings were incubated in X-Gluc and subsequently destained in 70 % (v/v) ethanol. GUS activity is visible as a dark contrast. A, Germinated conidium (C) with germ tube (G) and several runner hyphae (R) on the lower epidermis of the tomato leaf are visible as well as penetration of a stoma (S) by penetration hyphae (P). B, After penetration of the leaf, a long hypha (H) is visible in the intercellular space between the mesophyll cells (M). C, Abundant growth of hyphae (H) can be seen near vascular tissue (V). D, High GUS activity is visible in the branched hyphae (H) near vascular tissue E, Around 12 days after inoculation, conidiophores (O) emerge from stomata (S). F, The conidiophores eventually produce conidia (C) on the lower side of the leaf. The transformants shown are ecp1-6 at 6 days after inoculation (A-C), ecp2-40 at 8 and 10 days after inoculation (D and E, respectively), and ecp1-21 at 16 days after inoculation (F). Bar = 25 μm.


B
C
D

transformants of *C. fulvum* (Table 1). In the P*ecp*1-*Gus* transformants, no GUS activity was observed in conidia, germ tubes, and runner hyphae on the surface of inoculated tomato leaves at early stages of infection (Fig. 5, A). However, in the P*ecp*2-*Gus* transformants, several conidia, germ tubes, and runner hyphae on the leaf showed a low level of GUS activity (Table 1). Once the fungus had entered the tomato leaf through the stomata around 4 days post inoculation, an increase in GUS activity in the thickened hyphae during growth in the intercellular space was observed for both P*ecp*1-*Gus* (Fig. 5, B) and P*ecp*2-*Gus* transformants (results not shown). Between 5 and 10 days post inoculation abundant fungal growth was observed near vascular tissue, which was correlated with high GUS activity in both the P*ecp*1-*Gus* transformants (Fig. 5, C) and the P*ecp*2-*Gus* transformants (Fig. 5, D). Ten days post inoculation, young conidiophores originating from the substomatal primordia and emerging through stomata showed high GUS activity (Fig. 5, E), while the newly formed conidia showed much less GUS activity or none at all, as can be seen in P*ecp*1-*Gus* transformants (Fig. 5, F). The transformant of *C. fulvum* containing the *uidA* gene controlled by the constitutive *gpd* promoter showed a consistently high level of GUS activity in conidia and during all growth stages *in planta* (results not shown), confirming that the *uidA* gene is constitutively expressed and that the availability of substrate (X-Gluc) was not a limiting factor in the GUS assays.

Discussion

Here we describe the isolation of the fungal protein ECP2 and localization of both ECP1 and ECP2, which are produced by *C. fulvum* during pathogenesis on tomato. The proteins were purified from apoplastic fluid originating from *C. fulvum*-infected tomato leaves. Upon anion exchange chromatography ECP2 eluted in two different fractions which after analysis by SDS-PAGE were shown to contain one and the same protein. ECP2 likely occurs in two conformations, a dimeric and a monomeric form, which are in equilibrium.

The localization of ECP1 and ECP2 proteins and *in situ* expression of *ecp*1 and *ecp*2 genes during pathogenesis were studied to obtain further information about a possible role of these proteins in the interaction between *C. fulvum* and tomato. Immunolocalization experiments revealed that ECP1 and ECP2 accumulated predominantly in extracellular material in close contact with fungal and host cell walls at late stages of infection. Compared to the accumulation of the ECPs in the apoplast, less accumulation of ECP1 and ECP2 was detected in the fungal hyphae themselves. Therefore, these proteins must be released from fungal hyphae into the intercellular space. Most likely, this is achieved via secretion as predicted from the presence of a signal sequence in the pre-protein of both ECP1 and ECP2 (Van den Ackerveken *et al.*, 1993). Apart from ECP1 and ECP2, also tomato PR-1b, unesterified pectin, and arabinogalactan proteins were detected in this extracellular material

by immunogold labeling (J.P. Wubben, unpublished), indicating that it consists of a complex network of fungal and plant macromolecules.

Previously, GUS assays have been used to follow growth of *C. fulvum in planta* (Oliver *et al.*, 1993; Roberts *et al.*, 1989). Here, transformants of *C. fulvum,* containing fusions of the promoter of *ecp*1 or *ecp*2 with the coding region of the *Gus* reporter gene (*uidA*) were used to study the expression of *ecp*1 and *ecp*2 during colonization of tomato. The transformants with intact insertions of P*ecp-Gus* constructs all showed induced GUS activity *in planta*. No GUS activity was found in transformants which lacked the P*ecp-Gus* constructs. Expression studies, using northern blot analyses, revealed that P*ecp*1-*Gus* and P*ecp*2-*Gus* transformants accumulated *uidA* transcripts at levels similar to those of the *ecp*1 and *ecp*2 transcripts in untransformed race 5 of *C. fulvum*. Therefore, GUS activity of P*ecp*1-*Gus* and P*ecp*2-*Gus* transformants *in planta* is a representative measure of *ecp*1 and *ecp*2 expression during pathogenesis.

The histochemical GUS assays as performed with X-Gluc staining on tomato seedlings inoculated with the P*ecp-Gus* transformants clearly demonstrated that transcription of *ecp*1 and *ecp*2 does not increase immediately after the fungus has entered the leaf through stomata. This is in contrast to transcription of the avirulence gene *avr*9 of *C. fulvum*, which starts immediately after the fungus penetrates a tomato leaf (Van den Ackerveken *et al.*, 1994). Increased expression of *ecp*1 and *ecp*2 is observed especially when the fungus is growing near vascular tissue. The high expression could be due to a high metabolic activity of the fungus in that area of the plant. However, most likely specific environmental conditions in the intercellular space, or particular plant signals are required to initiate transcription of the *ecp*1 gene and to increase transcription of the *ecp*2 gene, since no such high levels of expression were found when the fungus was grown *in vitro*. As in *Rhizobium*- and *Agrobacterium*-plant interactions (Peters *et al.*, 1986; Stachel *et al.*, 1985), plant factors might be involved in the activation of the *ecp* genes. Low nitrogen concentration was found to trigger the expression of avirulence gene *avr9 in vitro* (Van den Ackerveken *et al.*, 1994) but did not induce *ecp*1 or *ecp*2 gene expression (G.F.J.M. Van den Ackerveken, unpublished). Since *ecp*1 and *ecp*2 gene expression was low in runner hyphae on the leaf and in newly formed conidia, a role for these proteins during pathogenesis is likely to be restricted to intercellular growth in tomato leaves. It is known that upon infection, the fungal biomass increases substantially and leaves develop symptoms of nutrient limitation. *C. fulvum* influences the carbohydrate metabolism of the tomato plant (Joosten *et al.*, 1990b) and withdraws sugars and other nutrients from tomato leaves primarily near vascular tissue, the area where the expression of *ecp*1 and *ecp*2 is highest.

The nucleotide sequences and amino acid sequences of ECP1 and ECP2 did not reveal known enzymatic or structural functions (Van den Ackerveken 1993). However, a significant similarity was observed in a repeat pattern of cysteines in ECP1 and tumour necrosis factor

receptors from mammalian systems (Bazan 1993). This author suggested a role for ECP1 as a soluble receptor, of which the host target might be a protein mediator of the plant defence response (Bazan 1993), a hypothesis which is currently under investigation.

Although the exact role of ECP1 and ECP2 during pathogenesis of *C. fulvum* on tomato remains to be elucidated, the localization of the proteins and the high level of expression of their encoding genes *in planta* suggest functions related to the growth of the fungus inside the tomato leaf. Presently, experiments are in progress in which the *ecp*1 and *ecp*2 genes will be disrupted separately or in combination, in order to study their role in pathogenicity.

Materials and methods

Purification of ECP2 and production of polyclonal antibodies

For the purification of ECP2, 300 ml of apoplastic fluid was isolated from a compatible interaction between tomato genotype Cf5 and race 5 of *C. fulvum* at 14 days after inoculation. Proteins were precipitated by adjusting the apoplastic fluid to 60 % (v/v) acetone, followed by incubation at -20°C for 3 h and centrifugation at 1,000 *g* for 5 min. The pellet was air-dried and resuspended in 7.5 ml of elution buffer (50 mM Tris-HCl and 100 mM NaCl, pH 7.5). After centrifugation for 10 min at 1500 *g* the proteins present in the supernatant were subjected to gel filtration on Sephadex G-50 column as described previously (Joosten *et al.*, 1990a). Fractions were combined according to the UV absorption profile (280 nm) of the eluate and analyzed by SDS-PAGE. Fractions containing the 17 kD ECP2 protein were deloused against H_2O, freeze-dried and dissolved in 30 mM Tris-HCl buffer, pH 8.5 (buffer A). Aliquots of 200 μl, containing about 3 mg of protein, were subjected to high resolution liquid chromatography (Bio-Rad, Richmond, CA) on a MA 7Q anion exchange column (Bio-Rad) which was equilibrated with buffer A. After the sample was loaded, the column was washed for 3 min with buffer A at a flow rate of 1.5 ml min^{-1}, and the bound proteins were subsequently eluted in buffer A, with a linear NaCl gradient from 0 to 200 mM in 15 min, followed by a linear gradient from 200 to 400 mM in 2.5 min, at a flow rate of 1.5 ml min^{-1}. Fractions of 0.75 ml were collected and the samples were analyzed by SDS-PAGE and native high pH PAGE as described by Joosten *et al.*, (1990a). Fractions containing ECP2 were freeze-dried, dialysed against H_2O, and rechromatographed under similar conditions, except that 30 mM Tris-HCl, pH 9.3, was used. This resulted in about 300 μg of pure ECP2. Polyclonal antibodies against the ECP2 protein were raised in rabbits as described previously (Joosten and De Wit 1988).

Immunogold localization of ECP1 and ECP2

Immunolocalization experiments were performed as described previously (Wubben *et al.*,

1992). Leaf discs of *C. fulvum*-inoculated tomato, obtained at 7 and 12 days post inoculation were fixed with formaldehyde and glutaraldehyde and subsequently embedded in LR Gold resin (Agar Scientific, Stansted, England) at low temperature (-25°C). Ultrathin sections were cut and incubated with antibodies. Detection of the antibodies was performed with protein A-gold (7 nm). For each experiment, 10 to 20 sections of five leaf samples each were labelled. The sections were stained with uranyl acetate and lead citrate and were then examined with a Philips CM 12 transmission electron microscope at 80 kV.

Construction of ecp*1 and* ecp*2 promoter-*GUS *fusions*

All recombinant DNA techniques were essentially performed according to Sambrook *et al.*, (1989). Fusions were made between promoter sequences of *ecp*1 or *ecp*2 and the *Gus* (*uidA*) reporter gene to study the expression of these genes in *C. fulvum* during colonization of tomato (Fig. 3). For both constructs, a fragment of at least 2 kb, upstream of the ATG startcodon of the gene was used.

For the *ecp*1 promoter (Fig. 3, A), a 1.8-kb *Eco*RV/*Xho*I fragment of pCF140, containing the *ecp*1 gene (Van den Ackerveken *et al.*. 1993), was subcloned in pBluescript II SK+ (Stratagene, La Jolla, CA). An *Nco*I site was introduced at the ATG startcodon of the gene with PCR using primer 1 (5'CGGGATCCATGGTGGAGGGAAGTGGG 3'), which contains an additional *Bam*HI site at the 5' end, and the T3 primer of pBluescript II SK+. The PCR product was digested with *Bam*HI, and the resulting fragment of 0.8 kb was subcloned in pGEM-3Zf(+) (Promega, Madison, WI). A *Pvu*II/*Eco*RV fragment of the *ecp*1 promoter region from pCF140 was subcloned at the *Eco*RV site of the PCR fragment which resulted in the reconstruction of a 2.2-kb *ecp*1 promoter fragment in pGEM-3Zf(+) (pCF150). The *gpd* promoter in front of the *uidA* gene in pCF20 was replaced by the *ecp*1 promoter fragment (Van den Ackerveken *et al.*, 1994), using the *Eco*RI and *Nco*I sites (pCF151). Cloning of the *ecp*2 promoter fragment in front of the *uidA* coding region was performed essentially the same as for the P*ecp*1-*Gus* construct (Fig. 3, B). A 2.9-kb *Hin*dIII/*Bam*HI fragment of pCF170 containing the *ecp*2 gene (Van den Ackerveken *et al.*, 1993) was subcloned in pGEM-3Zf(+). An *Nco*I site was created at the ATG startcodon of the gene with PCR using primer 2 (5'CGGGATCCATGGTGAAGCTGGTTGTATGG 3') and the T7 primer from pGEM-3Zf(+). A 2.3-kb PCR fragment was subcloned in a *Sma*I digested pGEM-3Zf(+) vector (pCF180) as described by Holton and Graham (1991). The *gpd* promoter in front of the *uidA* gene in pCF20 was subsequently replaced by the 2.3-kb *Eco*RI/*Nco*I promoter fragment of *ecp*2 (pCF181).

Fungal protoplast isolation and transformation

Isolation of protoplasts and transformation of *C. fulvum* were performed according to the

procedures described by Harling *et al.* (1988) and Oliver *et al.* (1987) with the modifications of Van den Ackerveken *et al.* (1992). Cotransformation of *C. fulvum* was performed by adding 4 μg of DNA (*ecp* promoter-*Gus* fusion constructs pCF151 and pCF181) and 2 μg of pAN7-1 as a selection marker (Punt *et al.*, 1987) to protoplasts of race 5 of *C. fulvum*.

GUS activity assays

GUS activity assays were performed as described by Jefferson *et al.*, (1987). In transformants, GUS activity was assayed on conidia germinated and grown on filter paper placed on potato-dextrose agar. The filter paper with germinated conidia was incubated overnight with X-Gluc (0.5 mg ml^{-1}) (Clontech, Palo Alto, CA) in 50 mM phosphate buffer pH 7.0, 1 mM KFeCN, and 0.05 % (v/v) Triton-X100, and GUS activity was estimated visually by the development of blue colour in the colonies. GUS activity of the fungus grown *in planta* was determined in homogenized leaf tissue. Ten volumes (v/w) extraction buffer (50 mM NaH_2PO_4 pH 7.0, 10 mM EDTA, 0.1 % [v/v] Triton-X100, 0.1 % [w/v] SDS, 10 mM ß-mercaptoethanol) were added to the infected leaflets and the material was homogenized with a pestle in a 1.5 ml reaction vial. After centrifugation for 1 min at 15,000 *g*, 25 μl of the supernatant was transferred to a single well of a 96-well microtiter plate. Aliquots of 25 μl of 2 mM 4-methylumbelliferyl glucuronide (Research Organics, Cleveland, Ohio) in extraction buffer were added to each well, and the plate was incubated at 37°C for 2 h. GUS activity was estimated visually on a UV transilluminator. Both these activity assays were used only to screen for co-transformation and to select for transformants which showed induced GUS activity *in planta*. Transformants, containing the *Gus* gene fused to the constitutive *gpd* promoter were used as an internal standard.

Southern blot analysis

DNA was extracted from freeze-dried mycelium as described by Van Kan *et al.* (1991). Five μg of DNA was digested with restriction endonucleases, separated on a 1 % agarose gel and blotted onto Hybond-N^+ (Amersham, 's-Hertogenbosch, Netherlands) by alkali blotting. Filters were hybridized with random-primed, ^{32}P-labelled DNA fragments (Hodgson and Fisk 1987). For P*ecp*1-*Gus* transformants the blot was hybridized with a probe containing a 1.4-kb *Eco*RV/*Hin*dIII *uidA-trpC* fragment or a 0.4-kb *Eco*RI/*Hin*dIII *ecp*1 promoter fragment. For P*ecp*2-*Gus* transformants a 2.9 kb *Eco*RI/*Eco*RV fragment of pCF181, containing a part of the promoter of *ecp*2 and a part of the coding sequence of *uidA*, was used as a probe.

Northern blot analysis

RNA was isolated from freeze-dried mycelium of *C. fulvum* and *C. fulvum* infected seedlings as described by Van Kan *et al.* (1991). A 10 μg sample of total RNA was separated on a 1.5 % denaturing formaldehyde-agarose gel and transferred onto Hybond-N^+ membranes as

described by Sambrook *et al.* (1989). Filters were hybridized with random-primed, ^{32}P-labelled DNA fragments obtained from *ecp*1 and *ecp*2 cDNA clones and *uidA* and actin genomic clones.

Histochemical ß-glucuronidase assay

Primary leaves of the inoculated seedlings were infiltrated at reduced pressure with X-Gluc (0.5 mg ml^{-1}) in 50 mM phosphate buffer, pH 7.0, 1 mM KFeCN and 0.05 % (v/v) Triton-X100, and incubated overnight at 37°C. The tissue was, subsequently, decolorized with 70 % (v/v) ethanol and mounted on microscope slides in 50 % (v/v) glycerol in phosphate-buffered saline. For histochemical GUS assays two individual transformants of each construct were used, respectively, for inoculation of leaves of six 3-wk-old tomato seedlings. The experiment was repeated two times. The stained leaves were examined with a Zeiss Axioscope, microscope and photographs were taken using a Zeiss MC-100 camera unit.

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chapter 8

General discussion

In the introduction of this thesis, several proteins of plant and fungal origin have been described which accumulate in the intercellular space of tomato leaves after inoculation with *Cladosporium fulvum*. These proteins are considered to be involved in different aspects of the infection process. The plant proteins belong to different classes of pathogenesis-related (PR) proteins (De Wit *et al.*, 1986; Joosten and De Wit, 1989; Joosten *et al.*, 1990). One class of fungal proteins detected in the apoplast of the inoculated leaf was identified as race-specific elicitors (Joosten *et al.*, 1994a; Van den Ackerveken *et al.*, 1992). Another class of fungal proteins supposedly plays a role in the infection process by establishing and maintaining basic compatibility (Joosten and De Wit, 1988; Van den Ackerveken *et al.*, 1993). In this thesis we have reported on additional characteristics of proteins accumulating in the apoplast of *C. fulvum*-infected tomato leaves and explored their possible roles in the infection process. Therefore, accumulation of these proteins in time and space, and the expression pattern of the corresponding genes have been determined by different histological methods.

ECP1 and ECP2

Immunocytochemical localization of infection-related extracellular fungal proteins, ECP1 and ECP2, revealed a strict accumulation of these proteins in extracellular material between fungal and host cell walls (Chapter 7). Expression studies using the ß-glucuronidase (*Gus*) reporter gene, revealed localized activation of *ecp*1 and *ecp*2 promoters during growth of *C. fulvum* in the intercellular space of the tomato leaf, especially near the vascular tissue (Chapter 7). These findings suggest that ECP1 and ECP2 could play a role in the infection process, possibly involved in uptake of nutrients or suppression of host defence responses. Based on amino acid sequence homology for ECP1 with a cysteine repeat pattern, present in tumour necrosis factor receptors, a role in suppression of the host defence response is suggested (Bazan, 1993). Gene disruption experiments of *ecp*1 and *ecp*2 are currently accomplished to obtain additional information on the role of these proteins in the infection process.

AVR4 and AVR9

Two race-specific elicitors, AVR4 and AVR9, have been isolated and identified in the apoplast of *C. fulvum*-infected tomato leaves. Immunolocalization experiments showed the accumulation of AVR4 and AVR9 in *C. fulvum*-infected tomato leaves in extracellular material around fungal hyphae near host cell walls, analogous to ECP1 and ECP2 (J.P.Wubben *et al.*, unpublished results). Expression studies performed with a transgenic race of *C. fulvum* containing the *Avr*9 promoter fused to the *Gus* reporter gene, revealed an activation pattern comparable to that observed for the *ecp*1 and *ecp*2 promoters (Van den Ackerveken *et al.*, 1994). However, instantaneous activation of the *Avr*9 promoter was

observed upon penetration *via* the stomata (Van den Ackerveken *et al.*, 1994), whereas *ecp*1 and *ecp*2 promoters were induced later during intercellular growth of *C. fulvum* (Chapter 7). Besides the induction of the hypersensitive response by race-specific elicitors, an intrinsic role of AVR4 and AVR9 for *C. fulvum* remains to be resolved.

PR-1 proteins

Detailed studies on protein localization and gene expression of different PR proteins accumulating in the apoplast of *C. fulvum*-infected tomato leaves have been performed. PR-1 proteins were found to accumulate in extracellular material which also contained substantial amounts of fungal proteins, such as ECP1, ECP2, and race-specific elicitors AVR4 and AVR9 (Chapter 4). Involvement of PR-1 proteins in plant cell wall modifications, triggered by fungal infection as reported by several researchers (Benhamou *et al.*, 1991; Tahiri-Alaoui *et al.*, 1993), could not be confirmed in this study. PR-1 proteins have been shown to contribute to active defence of plants against oomycetous pathogens (Niderman *et al.*, 1993, Alexander *et al.*, 1993). However, a role for PR-1 in defence of tomato against *C. fulvum* is still unclear.

PR-2 and PR-3 proteins; 1,3-ß-glucanases and chitinases

PR-2 (1,3-ß-glucanase) and PR-3 (chitinase) proteins accumulated at similar locations in *C. fulvum*-infected tomato leaves (Chapter 2 and 3). Specific accumulation of 1,3-ß-glucanases and chitinases has been observed near stomatal guard cells of the lower epidermis in *C. fulvum*-infected tomato leaves (Chapter 2). Further detailed studies revealed accumulation of 1,3-ß-glucanase and chitinase primarily in vacuoles of mesophyll cells, and in the intercellular space associated with extracellular material around fungal hyphae or between tomato mesophyll cells (Chapter 3). Specific association of 1,3-ß-glucanase and chitinase with fungal cell walls has not been observed, suggesting that these plant hydrolytic enzymes do not directly interact with their potential substrates in cell walls of *C. fulvum*. Support for this hypothesis was found by the observation that chitin in cell walls of *C. fulvum* is embedded in an electron-dense layer and hence inaccessible for plant chitinases. Differences in chitin distribution in cell walls of hyphae of *C. fulvum* grown *in vitro* or *in planta* could not be observed (Chapter 5). Surprisingly, only temporal but no spatial differences in accumulation of PR-2 and PR-3 proteins have been observed, when comparing compatible to incompatible *C. fulvum*-tomato interactions. These results could be confirmed by *in situ* hybridization in *C. fulvum*-infected tomato leaves. Temporal differences in gene transcript accumulation could be observed only for the acidic, extracellular isoforms of 1,3-ß-glucanase and chitinase, but not for their basic, intracellular counterparts (Chapter 6). Expression of the extracellular, acidic isoforms of 1,3-ß-glucanase and chitinase was restricted to vascular tissue and epidermal cells, whereas gene expression of intracellular, basic isoforms was not limited to

particular tissues (Chapter 6). No preferential accumulation of transcripts was observed near penetrating fungal hyphae.

The immunolocalization experiments do not provide evidence for an active role for extracellular 1,3-ß-glucanase and chitinase in defence of tomato against *C. fulvum*. Whether temporal differences in extracellular PR protein accumulation account for resistance of tomato towards *C. fulvum*, is unknown. In addition, Joosten *et al.* (1994b) found that these proteins failed to inhibit growth of *C. fulvum in vitro*. In conclusion, these observations do not support an important role of chitinase and 1,3-ß-glucanase in active defence of tomato against *C. fulvum*. The differential accumulation of several apoplastic PR proteins in compatible and incompatible interactions between *C. fulvum* and tomato is probably only part of a framework of plant defence responses induced earlier in incompatible interaction than in compatible ones, and which could be regarded as an indicator of incompatibility.

Induction of PR gene expression by race-specific elicitors

In addition, the involvement of race-specific elicitors, AVR4 and AVR9, in activating the plant defence response has been examined, focused on the induction of PR gene expression. Acidic chitinase and 1,3-ß-glucanase gene transcripts accumulated in a resistance gene-dependent manner upon injection of purified race-specific elicitors AVR4 and AVR9, in tomato genotypes Cf4 and Cf9, respectively (Chapter 6). This indicates that the race-specific elicitors are responsible for the differences in chitinase and 1,3-ß-glucanase gene expression as previously observed between compatible and incompatible interactions of *C. fulvum* and tomato (Danhash *et al.*, 1993; Van Kan *et al.*, 1992). Furthermore, preliminary results indicate that distribution of PR gene expression induced by pure race-specific elicitors is similar to induction by *C. fulvum* (J.P. Wubben, unpublished). This suggests that cells respond to specific inducing signals with activation of genes encoding extracellular 1,3-ß-glucanases and chitinases. Induced expression of genes encoding extracellular PR proteins in compatible interactions between *C. fulvum* and tomato is most likely the result of non-specific stress caused by abundant fungal growth in intercellular spaces and possibly production of non-specific elicitors.

PR proteins and resistance of plants against pathogens

Growth inhibition of *C. fulvum* in resistant tomato leaves is probably the result of the early induction of a hypersensitive response (HR), combined with other plant defence responses, such as the accumulation of reactive oxygen species (Vera-Estrella *et al.*, 1992). The importance of induced expression of single PR genes in defence of tomato against *C. fulvum* is probably limited. Several observations have been made recently, indicating that constitutive expression of a single PR gene can result in increased resistance against some pathogenic fungi (Alexander *et al.*, 1993; Broglie *et al.*, 1991; Liu *et al.*, 1994; Van den Elzen *et al.*,

1993). Furthermore, *in vitro* antifungal activity of purified PR proteins against several fungi, has been observed (Hejgaard *et al.*, 1992; Mauch *et al.*, 1988; Ponstein *et al.*, 1994; Sela-Buurlage *et al.*,1993; Vigers *et al.*, 1991 and 1992; Woloshuk *et al.*, 1991). Combined application of different PR proteins resulted in a synergistic increase of antifungal activity towards different fungi (Mauch *et al.*, 1988; Sela-Buurlage *et al.*,1993). Induced accumulation of a single PR protein will therefore only result in increased resistance towards a limited number of pathogens. Activation of a wide range of defence responses will extend the efficiency of the resistance reaction towards a broader range of pathogens. This idea is supported by research on systemic acquired resistance (SAR) against various pathogens, which is correlated with concomitant induction of a whole range of defence-related proteins, including PR proteins (Ward *et al.*, 1991).

Programmed defence responses

Research on induction of defence responses in plants resulted recently in important new insights on programmed cell death in plant-pathogen interactions. Two groups, independently, described the characterization of ethyl methane sulphonate (EMS) mutants of Arabidopsis, which spontaneously form necrotic lesions on leaves (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994). These mutants revealed an HR-like response in the absence of pathogens. In one occasion, the mutant phenotype was caused by a mutation in a single accelerated cell death (*ACD*) gene, *ACD2*. In addition to the HR-like response, other characteristic plant defence responses have been observed in the mutant plants, including modification of cell walls, accumulation of defence-related gene transcripts, accumulation of the signal molecule salicylic acid, accumulation of an antimicrobial compound, and resistance to a normally virulent bacterial pathogen. It has been suggested that HR is part of a multifaceted defence response, which represents a genetically defined programmed cell death, triggered by an environmental signal. This response is most probably negatively regulated by the product of the *ACD2* gene.

Activation of plant defence responses

The hypothesis, describing HR as part of genetically defined programmed cell death does not provide further information on molecular mechanisms involved in activation of plant defence responses upon challenge by pathogenic organisms. One of the molecules involved in induction of plant defence responses is salicylic acid (SA). Transgenic plants harbouring a bacterial gene encoding salicylate hydrolase, converting salicylic acid into catechol, were defective in their ability to induce systemic resistance against tobacco mosaic virus (Gaffney *et al.*, 1993). Increase in primary lesion size in these transgenic plants, after TMV infection, confirms a role for SA in local defence responses (Gaffney *et al.*, 1993). Results obtained by Rasmussen *et al.* (1991) and Vernooij *et al.* (1994) indicated that salicylic acid is not the

long distance signal involved in induction of systemic acquired resistance. Clues on the mode of action of salicylic acid in induction of plant defence responses were provided by the isolation and characterization of a salicylic acid binding protein (SABP) (Chen *et al.*, 1993a and 1993b) which appeared to be a catalase, an enzyme converting H_2O_2 in H_2O and O_2. Binding of SA to SABP led to inhibition of catalase activity, concomitant with accumulation of reactive oxygen species. Reactive oxygen species such as H_2O_2 have frequently been implicated in plant defence responses, either as activators of protein cross linking, as intrinsic antimicrobial compounds, or as signal transducers leading to gene activation (Sutherland, 1991). Further research on the involvement of salicylic acid and reactive oxygen species in induction of plant defence responses is required for further unravelling of these processes.

Studies on induced programmed cell death as described for *Arabidopsis thaliana* provide information on the induced framework of programmed defence responses. Different aspects of the framework of defence responses in a plant-pathogen interaction might have broader implications than resistance against the inducing pathogen only. Detailed studies on basic mechanisms involved in recognition and induction of defence responses may provide new insights which eventually could lead to alternative ways to control plant pathogens.

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Summary

In this thesis data are presented on the localization of gene expression and accumulation of differentially accumulating proteins in the interaction between the plant pathogenic fungus *Cladosporium fulvum* and tomato. Differential accumulation of proteins in the apoplast of *C. fulvum*-infected leaves in compatible and incompatible interactions initiated detailed studies on isolation and characterization of these proteins in order to establish their role in the infection process. On the one hand, plant proteins accumulating in the apoplast of *C. fulvum*-infected tomato leaves had been suggested to be involved in host defence responses. On the other hand, fungal proteins present in the apoplast of *C. fulvum*-infected tomato leaves had been suggested to play a role in establishment and maintenance of basic compatibility or in specific recognition events resulting in compatible or incompatible interactions.

Accumulation of pathogenesis-related (PR) plant proteins has been observed to occur 2-4 days earlier in incompatible interactions between *C. fulvum* and tomato than in compatible interactions. A significant role in plant defence against fungal pathogens was proposed for chitinases and 1,3-ß-glucanases. These PR proteins were identified as potential antifungal proteins due to their ability to hydrolyse chitin and 1,3-ß-glucan polymers present in fungal cell walls. Indeed it has been shown that these proteins could inhibit fungal growth *in vitro*.

In this thesis the localized accumulation of chitinases and 1,3-ß-glucanases in *C. fulvum*-infected tomato leaves has been described. Immunogold cytochemistry revealed PR protein accumulation near stomatal guard cells in the lower epidermis of *C. fulvum*-infected tomato leaves. Differential PR protein accumulation near stomata has not been observed between compatible and incompatible interactions. Accumulation of chitinases and 1,3-ß-glucanases near stomata can be regarded as a general defence response of plants against pathogens, which is induced to a similar extent in compatible and incompatible interactions.

More detailed localization studies of chitinases and 1,3-ß-glucanases in *C. fulvum*-infected tomato leaves have been carried out by electron microscope immunogold labelling. Specific accumulation of chitinases and 1,3-ß-glucanases has been observed in vacuoles of mesophyll cells and in the intercellular space in extracellular material present around fungal hyphae and between tomato mesophyll cells. Specific association of chitinases and 1,3-ß-glucanases with cell walls of *C. fulvum* has not been observed.

In addition to accumulation of the hydrolytic PR proteins, chitinases and 1,3-ß-glucanases, the spatial distribution of PR-1 protein accumulation in *C. fulvum*-infected tomato leaves has also been described. These proteins accumulate in the apoplast of *C. fulvum*-infected tomato leaves, primarily in extracellular material around fungal hyphae. Spatial differences in PR-1 accumulation between compatible and incompatible interactions have not been observed. A role for PR-1 proteins involved in cell wall modifications, possibly leading to resistance against pathogens, as has been reported previously, could not be supported in

this study. Apart from the localized accumulation near fungal hyphae, we did not obtain further information on a function for PR-1 proteins in the interaction between *C. fulvum* and tomato.

Cell wall modifications, potentially resulting from the activity of hydrolytic enzymes in the apoplast of *C. fulvum*-infected tomato leaves, have been examined indirectly by determining the distribution of substrates for these enzymes in cell walls of either the plant or the fungus using immunocytochemical labelling. In these studies probes specific for pectin compounds in plant cell walls or for chitin residues in fungal cell walls were used. Alterations in the distribution of unesterified pectin and methylesterified pectin in the plant extracellular matrix between *C. fulvum*-infected and uninfected tomato leaf tissue were not observed. However, some accumulation of unesterified pectin was observed in extracellular material near fungal hyphae. This accumulation might result from cell wall degrading enzymes of plant or fungal origin. Furthermore, differences in chitin distribution in cell walls of *C. fulvum* grown *in vitro* or *in planta* has not been observed. Chitin in cell walls of *C. fulvum*, is probably embedded in a matrix of amorphous material and therefore not accessible for chitinases that accumulate in the intercellular space of *C. fulvum*-infected tomato leaves.

Results on the spatial distribution of PR proteins in *C. fulvum*-infected tomato leaves, did provide further information on a role in defence of tomato against *C. fulvum*. In addition, northern analyses, revealed accumulation of transcripts encoding extracellular PR proteins, 2-4 days earlier in incompatible interactions than in compatible ones. Expression of genes encoding vacuolar PR proteins was induced to a similar extent in compatible and incompatible interactions. Spatial distribution of PR gene expression in tomato upon infection by *C. fulvum* was studied by *in situ* hybridization. Expression of the extracellular, acidic isoforms of chitinase and 1,3-ß-glucanase occurred near vascular tissue and near epidermal cells, whereas expression of intracellular, basic isoforms was less restricted to particular tissue. No preferential accumulation of transcripts has been observed near penetrating fungal hyphae. Temporal differences in accumulation of gene transcripts between compatible and incompatible interactions could only be observed for the acidic PR proteins, thus confirming the northern analyses carried out before.

Induced gene expression of acidic chitinases and 1,3-ß-glucanases has been observed in a resistance gene-dependent manner upon injection of purified race-specific elicitors, AVR4 and AVR9, in tomato genotypes, Cf4 and Cf9, respectively. Induced gene expression, primarily observed in resistant tomato genotypes, correlates with preferential induction of chitinase and 1,3-ß-glucanase gene expression in incompatible interactions as previously observed after inoculation by *C. fulvum*.

An significant role for hydrolytic PR proteins in active defence of tomato against *C. fulvum* could not be proven. Only temporal differences were observed in accumulation of acidic isoforms of chitinase and 1,3-ß-glucanase which, however, have been shown to possess only limited *in vitro* antifungal properties. Furthermore, non of the purified chitinases and

1,3-ß-glucanases did inhibit growth of *C. fulvum in vitro*. Therefore, it is unlikely that they play an important role in direct defence of tomato against *C. fulvum*.

Two extracellular fungal proteins, ECP1 and ECP2, have been described in the last section of this thesis. These proteins are produced by *C. fulvum* primarily during growth on tomato. Immunogold localization experiments revealed that ECP1 and ECP2 accumulated abundantly in extracellular material near fungal and host cell walls. Expression studies, using the ß-glucuronidase reporter gene, revealed induction of *ecp*1 and *ecp*2 expression only during growth of *C. fulvum* in intercellular spaces of tomato leaves. Fungal hyphae growing near vascular tissue in infected tomato leaves, showed high *ecp* gene expression. No *Ecp* gene expression was observed in conidia used for inoculation nor in newly formed conidia on infected plants. A function for ECP1 and ECP2 in pathogenesis of *C. fulvum* on tomato is not clear. However, the accumulation near extracellular material associated with fungal and host cell walls, and the induced expression of *ecp*1 and *ecp*2 *in planta* suggest a role for both genes during intercellular growth of *C. fulvum* in tomato.

Samenvatting

In dit proefschrift worden de resultaten beschreven aangaande de lokalisatie van eiwitten welke specifiek ophopen in de interactie tussen tomateplanten en het schimmelpathogeen *Cladosporium fulvum*. Van een aantal van deze eiwitten is eveneens het tijdstip en de plaats van genexpressie bestudeerd in geïnfecteerd tomateblad. Een vroege accumulatie van een aantal van deze eiwitten werd gevonden in de apoplast van tomatebladeren in een incompatibele interactie met *C. fulvum* (plant is resistent tegen de schimmel) in vergelijking met een compatibele interactie (plant is gevoelig voor de schimmel). Dit gaf aanleiding tot verder gedetailleerd onderzoek naar de zuivering en karakterisering van deze eiwitten, in relatie tot hun functie in het infectieproces. Een aantal planteneiwitten werd gevonden in de apoplast van geïnfecteerde tomatebladeren die mogelijk betrokken zijn bij de resistentie reactie van de plant. Verder accumuleerden ook schimmeleiwitten in de apoplast van geïnfecteerde tomatebladeren die betrokken kunnen zijn bij vestiging van *C. fulvum* in het tomateblad en in stand houden van basis-compatibiliteit. Een andere mogelijkheid is dat deze schimmeleiwitten een rol spelen in de specifieke herkenning van de schimmel door de plant waardoor een incompatibele interactie plaatsvindt.

Een aantal pathogenese-gerelateerde (PR) eiwitten afkomstig van de plant accumuleerden 2-4 dagen eerder in incompatibele interacties tussen *C. fulvum* en de tomateplant dan in compatibele interacties. Voor een aantal van deze PR eiwitten, chitinases en 1,3-ß-glucanases, is een belangrijke rol voorgesteld in afweer van planten tegen schimmelpathogenen. Chitinases en 1,3-ß-glucanases bezitten potentiële antischimmelactiviteit omdat zij in staat zijn belangrijke bestanddelen van celwanden van veel schimmels af te breken, namelijk chitine en 1,3-ß-glucanen. Dit werd bevestigd door de waarneming dat chitinases en 1,3-ß-glucanases de groei van schimmels *in vitro* kunnen remmen.

In dit proefschrift wordt de lokale accumulatie van chitinases en 1,3-ß-glucanases beschreven zoals gevonden wordt in tomateblad geïnfecteerd met *C. fulvum*. Met behulp van immunogoudlokalisatie is aangetoond dat deze PR eiwitten ophopen in huidmondjes van de onderepidermis van geïnfecteerde tomatebladeren. Echter, tussen compatibele en incompatibele interacties van *C. fulvum* en tomaat, konden geen verschillen waargenomen worden in de accumulatie van deze PR eiwitten in de huidmondjes. De plaatselijke ophoping van chitinases en 1,3-ß-glucanases rond de huidmondjes is mogelijk een onderdeel van een algemene afweerreactie van planten tegen schimmelpathogenen, waarvan de inductie in compatibele en incompatibele interacties tussen de tomateplant en *C. fulvum* gelijk is.

Gedetailleerde lokalisatie van chitinases en 1,3-ß-glucanases, in tomatebladeren geïnfecteerd met *C. fulvum*, werd uitgevoerd met behulp van immuno-elektronenmicroscopie. Specifieke accumulatie van chitinases en 1,3-ß-glucanases werd waargenomen in vacuolen van mesofylcellen en in extracellulair materiaal zowel rond schimmelhyfen als tussen

mesofylcellen. Ophoping van chitinases en 1,3-ß-glucanases in de celwand van *C. fulvum* is niet waargenomen.

Behalve het onderzoek aan chitinases en 1,3-ß-glucanases is eveneens gekeken naar de lokalisatie van PR-1 eiwitten in geïnfecteerde tomatebladeren. Deze eiwitten komen voor in de apoplast van geïnfecteerd blad, met name in extracellulair materiaal rond de hyfen van *C. fulvum*. In de lokalisatie van PR-1 eiwitten werd geen verschil waargenomen tussen compatibele en incompatibele interacties van tomaat en *C. fulvum*. Door diverse onderzoekers is eerder waargenomen dat PR-1 eiwitten in de afweerreactie van planten tegen schimmels mogelijk betrokken zijn in modificaties van de plantecelwand. In dit onderzoek kon deze waarneming niet bevestigd worden. Verdere indicaties met betrekking tot een functie van PR-1 eiwitten in de afweer van de tomateplant tegen *C. fulvum* werden niet verkregen.

Veranderingen in de celwand van plant en schimmel werden bestudeerd en als een indirecte maat voor de activiteit van celwand afbrekende enzymen in de intercellulaire ruimte van geïnfecteerd tomateblad genomen. Hiertoe werd met behulp van immuno-electronenmicroscopie gekeken naar de specifieke verdeling van pectine en chitine in, respectievelijk, de celwand van de plant en de celwand van de schimmel. De verdeling van gemethyleerd en niet-gemethyleerd pectine in celwanden van geïnfecteerde tomatebladeren was vergelijkbaar met de verdeling in celwanden van gezonde bladeren. Echter een lichte ophoping van niet-gemethyleerd pectine werd gevonden in extracellulair materiaal tussen de celwand van de plant en de celwand van de schimmel. Dit zou de aanwezigheid van pectine afbrekende enzymen afkomstig van plant of schimmel suggereren. De verdeling van chitine in de celwand van de schimmel was vergelijkbaar voor hyfen in het geïnfecteerde tomateblad en hyfen afkomstig van opkweek in kunstmatig medium. Chitine in de celwand van *C. fulvum* is waarschijnlijk omgeven door een matrix. Hierdoor is het chitine moeilijk bereikbaar voor de chitinases die in de intercellulaire ruimte van het geïnfecteerde tomateblad ophopen.

De gevonden verdeling van PR eiwitten in *C. fulvum*-geïnfecteerd tomateblad geeft informatie over een mogelijke functie van deze eiwitten in de afweerreactie. Genexpressie studies, welke eerder uitgevoerd waren met behulp van northernhybridisatie, toonden aan dat de transcripten voor zure extracellulaire PR eiwitten, 2-4 dagen eerder accumuleerden in incompatibele interacties dan in compatibele interacties. Expressie van de genen welke coderen voor basische intracellulaire PR eiwitten, werd gelijktijdig geïnduceerd in compatibele en incompatibele interacties. Met behulp van *in situ* hybridisatie is de lokalisatie van expressie van PR genen in *C. fulvum*-geïnfecteerde tomateplanten bestudeerd. De genexpressie van zure extracellulaire chitinases en 1,3-ß-glucanases vond voornamelijk plaats rond vaatweefsel en in cellen van de epidermis. De basische intracellulaire chitinases en 1,3-ß-glucanases vertoonden een meer gespreide genexpressie in verschillende celtypen. Geen verhoogde PR genexpressie kon worden waargenomen rond penetratieplaatsen van de schimmel. Alleen een verschil in tijd werd waargenomen tussen compatibele en incompatibele interacties van tomaat en *C. fulvum* voor genexpressie van extracellulaire PR genen. Dit verschil kwam overeen met de eerder gevonden resultaten van northernhybridisaties.

Met behulp van northernhybridisaties kon tevens worden aangetoond dat injectie van gezuiverde elicitoren, AVR4 en AVR9, in respectievelijk Cf4 en Cf9 genotypen van de tomateplant, voornamelijk de genexpressie van zure chitinases en 1,3-ß-glucanases aanschakelen. Deze specifiek geïnduceerde genexpressie komt overeen met de eerder gevonden resultaten in een tomaat geïnfecteerd met *C. fulvum*.

Een actieve rol van hydrolytische PR eiwitten in de afweerreactie van tomaat tegen *C. fulvum* kon in dit onderzoek niet worden bewezen. Alleen voor zure extracellulaire chitinases en 1,3-ß-glucanases werden verschillen in het tijdstip van accumulatie tussen compatibele en incompatibele interacties waargenomen. Echter, deze enzymen bezitten *in vitro* slechts beperkte antischimmelactiviteit. Verder is voor geen van de gezuiverde chitinases en 1,3-ß-glucanases van de tomateplant antischimmelactiviteit tegen *C. fulvum* waargenomen. Het is daarom niet waarschijnlijk dat deze PR eiwitten een belangrijke directe rol spelen in de resistentie van tomaat tegen *C. fulvum*.

In het laatste deel van dit proefschrift staat het onderzoek beschreven dat uitgevoerd is aan twee extracellulaire schimmeleiwitten (ECPs) welke door *C. fulvum* geproduceerd worden gedurende de infectie. Immuno-electronenmicroscopie toonde aan dat de eiwitten ECP1 en ECP2 met name voorkomen in extracellulair materiaal dat zich bevindt tussen de celwand van de schimmel en de celwand van de plant. Met behulp van het *Gus* reporter-gen zijn expressiestudies uitgevoerd waarbij inductie van promoteractiviteit alleen werd waargenomen gedurende de groei van *C. fulvum* in de intercellulaire ruimte van het tomateblad. Vooral wanneer de schimmel rond het vaatweefsel van de plant groeide werd een hoge promoteractiviteit voor beide *ecp*-genen waargenomen. Zowel in de sporen die gebruikt waren voor de inoculatie, alsmede in de nieuw gevormde sporen op het geïnfecteerde blad, werd geen *ecp* promoteractiviteit waargenomen. Een mogelijke functie voor ECP1 en ECP2 tijdens de infectie van *C. fulvum* op tomaat is nog niet bekend. Het voorkomen van deze eiwitten in extracellulair materiaal rond de celwanden van de schimmel en de plant, in combinatie met de geïnduceerde genexpressie gedurende groei van *C. fulvum* in de intercellulaire ruimte van het tomateblad, suggereren functies voor ECP1 en ECP2 die waarschijnlijk betrokken zijn met de groei van de schimmel in de intercellulaire ruimte van het blad.

Curriculum vitae

Josephus Petrus Wubben werd op 7 juni 1965 geboren in Naaldwijk. In 1983 behaalde hij het VWO diploma aan het Bonaventura College te Leiden. In datzelfde jaar ving hij aan met de studie Planteziektenkunde aan de Landbouwuniversiteit Wageningen alwaar hij in augustus 1989 afstudeerde. De doctoraal studie omvatte als afstudeervakken Moleculaire Biologie en Fytopathologie. Zijn stage bracht hij door bij professor Wolfram Köller, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York, van oktober 1987 tot april 1988. In november 1989 begon hij bij de vakgroep Fytopathologie met het promotie-onderzoek waarvan de resultaten staan beschreven in dit proefschrift. Vanaf 1 augustus is hij werkzaam bij het Sainsbury Laboratory, John Innes Centre, Norwich, Engeland.

Nawoord

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