Extracellular proteins of the tomato pathogen *Cladosporium fulvum*; role in pathogenicity and avirulence

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Stellingen

1. The parallel hypothesized in the early nineties between the structure and dynamics of the major histocompatibility complex in animals and the resistance genes in plants was a prediction that came true.
Dangl, J.L. (1992) Plant J. 2:3-11.

2. Our growing knowledge on resistance genes suggests the existence of a plant recognition system for exogenous proteinaceous signal molecules, which shares similarities with perception of endogenous signals involved in plant development and reproduction.
Becraft, P.W. (1998) Trends Plant Sci. 3:384-388; Nasrallah, J.B. (1997) in Essays in Biochemistry: Cell signalling , Bowles, D.J. ed., Portland Press, London-Miami.

3. The wide occurrence of gene-for-gene systems in crop plants is most probably a result of human plant breeding activities, involving transfer and dissection of gene clusters that originate from wild species and simultaneous co-evolution of pathogens.
This thesis.

4. After focusing on the cloning of molecular determinants of gene-for-gene combinations, efforts should be made to study the molecular and functional diversity of these determinants at the population and species levels to obtain a complete view of natural pathosystems.
This thesis.

5. Virus expression systems are invaluable tools in animal biology, veterinary sciences and medicine. They have now also become useful tools in plant biology and phytopathology.
Various authors (1996) Proc. Natl. Acad. Sci. USA 93:11287-11424; Yokoyama, N. et al. (1997) J. Vet. Med. Sci. 59:311-322; Chapman, S. et al. (1992) Plant J. 2:549-557; This thesis; Karrer, E.E. et al. (1998) Plant Mol. Biol. 36:681-690.

6. The existence of viral proteins subverting, counteracting, and suppressing defense responses of animal and plant hosts is simply amazing.
Gooding, L.R. (1992) Cell 71:5-7; Marrack, P., and Kappler, J. (1994) Cell 76:323-332; McFadden, G. (1998) Science 279:40-41; Brigneti, G. et al. (1998) EMBO J. 17:6739-6746; Kasschau, K.D., and Carrington, J.C. (1998) Cell 95:461-470; Anandalakshmi, R. et al. (1998) Proc. Natl. Acad. Sci. USA 95:13079-13084.

7. Resistance genes might rather be named "surveillance genes" as, similar to genes of the immune system, their products apparently exist in the absence of the matching elicitor from a pathogen.

8. The Dutch language is not tender with French sayings. First you hear about "een leven hebben als God in Frankrijk" and you get flattered, but then you hear about "met de Franse slag iets doen" and you change your mind.
Geerts, G., and Heestermans, H. (1995) Groot Woordenboek der Nederlandse Taal, van Dale, Utrecht-Antwerpen.

9. "Plus ça rate et plus on a de chances que ça marche". Philosophie Shadok.
Rouxel, J. (1994) Les Shadoks, éditions Circonflexe, Paris.

Stellingen behorende bij het proefschrift van Richard Laugé: Extracellular proteins of the tomato pathogen *Cladosporium fulvum*; role in pathogenicity and avirulence. Wageningen, 3 maart 1999.

Contents

Chapter 1

General introduction and outline of thesis

GENERAL INTRODUCTION

When a plant and a microbe encounter in nature, this can result in three different types of outcome (Agrios, 1997). The first and most common possibility is that the microbe lacks crucial "weapons" to attack and infect a plant species; there is absence of basic compatibility, the microbe is not a pathogen of the plant species and as a result no compatible interaction occurs between the two organisms (non-host resistance) (Heath, 1991). Secondly, the microbe possesses crucial "weapons" to attack and infect a plant species which fails to arrest the development of the pathogenic microbe; there is a compatible interaction resulting in disease (susceptibility). Thirdly, the microbe possesses crucial "weapons" to attack and infect a plant species of which, however, some individuals (lines or cultivars) arrest the development of the pathogenic microbe; there is an incompatible interaction and the individuals are resistant to the disease-causing microbe (genotype-specific resistance). The latter situation is observed for many plant-pathogen interactions. However, this phenomenon occurs more frequently for pathogenic microbes that exhibit a biotrophic phase during the disease cycle (e.g. part of the disease cycle takes place when host tissues are alive). As a corollary, within populations of host plants, besides the majority of individuals that are susceptible to the pathogen, there are few individuals that are resistant.

Early genetic studies with host plants proved that genotype-specific resistance is mostly monogenic and dominant. More recent genetic studies with pathogens showed that avirulence (e.g. the property of a strain of a pathogen to become recognized and arrested by the host carrying the resistance gene) usually also inherits as a monogenic and dominant character. The integration of both genetic studies led to the gene-for-gene model that states that for each plant resistance (R) gene that confers resistance to a given pathogen, there is a corresponding pathogen avirulence (Avr) gene that confers avirulence (Flor, 1946). A biochemical model, the elicitor-receptor model, was subsequently proposed that explains this gene-for-gene relationship through interaction of both R and Avr gene products (Gabriel and Rolfe, 1990; Keen, 1990; De Wit, 1992). According to this model a specific elicitor, the putative product of the pathogen Avr gene, is perceived through a specific receptor, the putative product of the corresponding host R gene.

Human societies have always cared for protection of their crops. Naturally occurring resistance traits have been extensively crossed into agronomically important cultivars of crops. The resulting protection has often been observed to be short due to appearance of new strains of the pathogen that could overcome the newly introgressed resistance genes. However, resistance breeding is still the most powerful tool to fight pathogens in an environmentally friendly way. With the advances of molecular techniques in biology and genetics, much emphasis has been put on the elucidation of the molecular basis of resistance. Besides the academic interest, the comprehensive understanding of

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how plants perceive and resist to a pathogen is crucial for resistance management and for the establishment of genetically engineered resistant crops.

Cladosporium fulvum (Cooke) (syn. *Fulvia fulva*, Ciferri), a fungus that belongs to the class of the Deuteromycetes, causes leaf mold disease on its only natural host, tomato (*Lycopersicon esculentum*, Miller). This pathogen is a typical biotroph that enters the leaf through stomata and grows exclusively in the intercellular space around mesophyll cells without causing detectable damage to these cells (De Wit, 1977). This plant-pathogen interaction complies with the gene-for-gene model and many independent resistance genes have been identified in wild *Lycopersicon* species (Day, 1974). As the fungus remains restricted to the intercellular space during colonization, all crucial molecules that are exchanged between the two organisms are hypothesized to be available through analysis of apoplastic fluids of infected plants (De Wit, 1992).

Research on this plant-fungus interaction resulted in the isolation and characterization of several pathogenesis-related (PR) proteins from tomato (Joosten and De Wit, 1989; Joosten *et al.*, 1990), as well as in the identification of two fungal extracellular proteins, ECP1 and ECP2, that are abundantly produced by *C. fulvum* during leaf colonization (Joosten and De Wit, 1988; Wubben *et al.*, 1994). Localization studies on the different classes of PR proteins revealed their extracellular accumulation in the vicinity of the invading fungus, suggesting a role in plant defense. However, purified chitinases and β-1,3-glucanases did not show antifungal activity against *C. fulvum* (Joosten *et al.*, 1995). The fungal proteins ECP1 and ECP2 accumulate in the extracellular matrix between the fungal hyphae and the plant cell wall, which suggests that these two proteins might play a role in pathogenicity or virulence of the fungus (Wubben *et al.*, 1994). However, a mutant strain where the *Ecp2* gene has been deleted appeared to be still pathogenic on tomato seedlings (Marmeisse et al., 1994).

Furthermore, two specific proteinaceous elicitors, AVR4 and AVR9, also secreted in the apoplast by *C. fulvum*, were proven to be the products of the two avirulence genes, *Avr4* and *Avr9*, that match the tomato resistance genes *Cf-4* and *Cf-9*, respectively (Van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). These two proteins trigger a specific hypersensitive response (HR), leading to cell death, when infiltrated in leaves of the matching resistant genotypes. This specific recognition accounts for the micro lesions that occur at the site of penetration of the leaf of a resistant genotype by an avirulent strain producing the functional matching avirulence product, and eventually stop further growth of the fungus. Fungal races virulent on tomato genotypes containing *Cf-4* or *Cf-9* were shown to become avirulent on these plants after transformation with the *Avr4* and *Avr9* gene, respectively (Van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). In addition, deletion of the *Avr9* gene in fungal races avirulent on *Cf-9* tomato plants resulted in mutant strains that had become virulent on *Cf-9* tomato plants (Marmeisse *et al.*, 1993).

OUTLINE OF THESIS

This thesis covers studies on the function of extracellular proteins (ECPs) in pathogenicity/virulence and avirulence of *C. fulvum*. We performed detailed studies on the role of ECP1 and ECP2 in pathogenicity/virulence. We searched for additional ECPs and their encoding genes. In addition to their potential role in pathogenicity/virulence, we studied their potential role in avirulence by screening tomato lines, *Lycopersicon* species and other solanaceous species, for their response to these proteins. At the same time, we studied their potential interaction with homologues of resistance genes of which the matching elicitors have already been identified.

Chapter 2 reviews the state of the art concerning fungal avirulence genes, including their structure, regulation, possible intrinsic functions and the suggested mechanisms of perception by the plant. Only a few fungal *Avr* genes have been cloned and fully characterized. They appear to be very diverse in structure and regulation and some have been demonstrated to be involved in pathogenicity or virulence.

In Chapter 3, the role of two extracellular proteins, ECP1 and ECP2, in pathogenicity/virulence of *C. fulvum* on adult tomato plants is described. *Ecp1*- and *Ecp2*-replacement mutants were made by homologous recombination and subsequently compared to the isogenic wild-type strain. No different phenotype was observed *in vitro*, but when inoculated onto plants, both mutants showed reduced fitness. Therefore, ECP1 and ECP2 are virulence factors as they both contribute to full virulence of the fungus, although their absence does not abolish disease. When challenged by either of the two deficient strains, the plant defense response levels are highly induced when compared to infection by the wild-type strain, which suggests that these two ECPs might be involved in suppression of the host defense responses.

Both ECPs are cysteine rich, low molecular weight proteins that occur early and abundantly in the apoplast during the infection. In this respect they are similar to the two avirulence factors AVR4 and AVR9. Consequently, it was decided to study whether they could act as avirulence factors on particular tomato genotypes. In Chapter 4, a search for tomato genotypes showing a HR upon exposure to ECP1 or ECP2 is described. Several breeding lines sharing a common ancestor exhibited HR to ECP2. The resistance of this tomato genotype to *C. fulvum* has been shown to rely solely on the recognition of ECP2, and is determined by a monogenic dominant character. Particularly, as described in Chapter 3, ECP2-deficiency causes *C. fulvum* to colonize the leaf mesophyll poorly and to sporulate scarcely at the end of the disease cycle. Therefore, the identified resistance gene might prove to be durable, as the fungus cannot overcome it without losing its pathogenic abilities.

The successful identification of a tomato genotype that specifically recognizes a targeted protein secreted by the fungus, prompted us to test whether additional ECPs could also be recognized as specific elicitors of HR by other tomato genotypes or *Lycopersicon*

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species. Chapter 5 presents the results of a search for plants exhibiting specific HR-associated recognition of four additional ECPs of *C. fulvum* that are produced during infection. Several breeding lines, as well as accessions of wild *Lycopersicon* species, were identified that respond with HR to the purified ECPs. This observation suggests the presence of an efficient and versatile surveillance system in the *Lycopersicon* genus, directed against proteins produced by *C. fulvum*. Moreover, accessions of *Nicotiana* spp. were identified that exhibit HR when exposed to ECP2. The latter result indicates that recognition of pathogen-derived molecules is not restricted to the host plant itself.

Meanwhile, the cloning and sequencing of the four tomato *Cf* genes, *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* had been reported (Hammond-Kosack and Jones, 1996; Jones and Jones, 1996). They all share extensive sequence and structure homology, although they operate through specific recognition of AVR4 and AVR9 (*Cf-4* and *Cf-9*, respectively), and two yet to be identified avirulence factors, AVR2 and AVR5 (*Cf-2* and *Cf-5*, respectively). These *Cf* genes were shown to be organized in clusters of homologous genes on chromosome 1 and 6 of the tomato genome. The genomic organization and the versatility of recognition specificities that we found for *C. fulvum* proteins, prompted us to investigate whether the clustered homologues of a characterized resistance gene could also confer race-specific resistance against the same organism but targeted against a different yet to be identified elicitor. In Chapter 6, we describe the characterization of a partial resistance trait located on the *Cf-9* introgression segment, that is independent of the *Avr9/Cf-9* gene-for-gene pair. Consistent with our hypothesis, similar work in another research laboratory demonstrated that two homologues of the *Cf-9* resistance gene, present at the *Cf-9* locus, are responsible for the observed partial resistance. We, however, did not yet find extracellular fungal proteinaceous elicitors that match the described homologues.

From this thesis research it can be concluded that recognition of nonself proteins by plants is not exceptional and is most probably a general strategy employed by plants to fight their pathogens at the population level. In Chapter 7, molecular, ecological and evolutionary aspects of such a strategy are discussed, as well as their potential relevance for molecular resistance breeding.

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Chapter 2

Fungal avirulence genes: structure and possible functions

REVIEW

Fungal Avirulence Genes: Structure and Possible Functions

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Laugé, R., and de Wit, J. G. M. 1998. Fungal avirulence genes: Structure and possible functions. *Fungal Genetics and Biology* **24,** 285–297. Avirulence (*Avr*) genes exist in many fungi that share a gene-for-gene relationship with their host plant. They represent unique genetic determinants that prevent fungi from causing disease on plants that possess matching resistance (*R*) genes. Interaction between elicitors (primary or secondary products of *Avr* genes) and host receptors in resistant plants causes induction of various defense responses often involving a hypersensitive response. *Avr* genes have been successfully isolated by reverse genetics and positional cloning. Five cultivar-specific *Avr* genes (*Avr4, Avr9,* and *Ecp2* from *Cladosporium fulvum;* nip1 from *Rhynchosporium secalis;* and *Avr2-YAMO* from *Magnaporthe grisea*) and three species-specific *Avr* genes (*PWL1* and *PWL2* from *M. grisea* and *inf1* from *Phytophthora infestans*) have been cloned. Isolation of additional *Avr* genes from these fungi, but also from other fungi such as *Uromyces vignae, Melampsora lini, Phytophthora sojae,* and *Leptosphaeria maculans,* is in progress. Molecular analyses of nonfunctional *Avr* gene alleles show that these originate from deletions or mutations in the open reading frame or the promoter sequence of an *Avr* gene. Although intrinsic biological functions of most *Avr* gene products are still unknown, recent studies have shown that two *Avr* genes, *nip1* and *Ecp2*, encode products that are important pathogenicity factors. All fungal *Avr* genes cloned so far have been demonstrated or predicted to encode extracellular proteins. Current studies focus on unraveling the mechanisms of perception of avirulence factors by plant receptors. The exploitation of *Avr* genes and the matching *R* genes in engineered resistance is also discussed. © 1998 Academic Press

Index Descriptors: avirulence; gene-for-gene; elicitor; cultivar specificity; species specificity; resistance; plant receptor; two-component system.

Many fungal plant diseases occur in nature. Unlike the limited number of species that cause disease in animals, several thousands of fungal species are responsible for plant diseases. It is known that every plant species is a potential host for various fungi. However, disease is an exception rather than a rule and most natural interactions between fungi and plants that live in the same biotope do not lead to a disease (Agrios, 1997). Most of these interactions are of the "nonhost" type, in which the fungus is not known to be pathogenic on a given plant, probably because the fungus lacks the basic pathogenicity factors that would enable it to cause disease on a plant (Heath, 1991). The remaining interactions are of the "host" type, in which the fungus is known to be a pathogen on a given plant. However, for the latter type of interaction, not all combinations of fungal strains and plant cultivars will lead

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to a disease. The host type of interactions are therefore divided into "compatible interactions" in which a "susceptible plant" becomes diseased upon attack by a "virulent pathogen" and "incompatible interactions" in which a "resistant plant" does not develop disease upon attack by an "avirulent pathogen." The first report of genetic plant resistance against a fungal disease goes back to the end of the last century when Farrer described resistance in wheat against yellow rust (Farrer, 1898). The resistant character was subsequently demonstrated to be a dominant monogenic trait (Biffen, 1905, 1907, 1912). In the following decades, several reports appeared on monogenic and polygenic resistances to various diseases in different plant species. Introgression into cultivars confirmed that monogenic resistances were usually dominant.

Disputes about whether specific interactions occur during compatible or incompatible interactions were finally resolved in the early forties. Flor, working on the *Melampsora lini*–flax interaction (Flor, 1946, 1955), and Oort, working on the *Ustilago tritici*–wheat interaction (Oort, 1944), made a genetic breakthrough by studying the genetics of both host plant and fungus. Heritability of virulence (the ability to cause a disease) and avirulence (the inability to cause disease on particular cultivars) was investigated. Flor made hybrids between strains of *M. lini* virulent or avirulent on a given flax cultivar and scored the resulting dikaryotic offspring. Virulence appeared to be recessive and avirulence dominant. This laid the basis for the gene-for-gene concept that states that for every dominant gene determining resistance in the host there is a matching dominant avirulence gene in the pathogen. This concept is illustrated in Table 1. The avirulence (*Avr*) gene of a pathogen exists by virtue of its matching resistance (*R*) gene in the host plant. Although gene-for-gene interactions have been described for many fungus–plant interactions, with examples from all classes of fungi, they occur most

frequently among obligate and biotrophic fungi (Day, 1974). While originally proposed for plant-pathogenic fungi, other pathogen–plant interactions involving bacteria, viruses, nematodes, and even insects have been shown to comply with the gene-for-gene concept (Agrios, 1997). The gene-for-gene concept has also been proposed to account for species-specificity in fungi that are known to infect several different plant species while individual strains are often restricted to one or few host plants. A few exceptions have been reported in which resistance and avirulence do not follow the typical inheritance patterns as described by Flor (1946). Examples include: (i) recessive inheritance of resistance and avirulence, (ii) resistance controlled by two genes, and (iii) additional control of resistance by modifiers or suppressors (Barrett, 1985; Christ *et al.*, 1987). Since the majority of *Avr* and *R* genes are dominant and likely to correspond to positive functions, the elicitor–receptor model has been proposed to explain the biochemical basis of the gene-for-gene concept (Gabriel and Rolfe, 1990; Keen, 1990; De Wit 1992, 1997). According to this model, a specific elicitor (putative product of an *Avr* gene) is recognized by a receptor (putative product of the matching *R* gene) of the resistant plant. This interaction would activate a signal transduction pathway that leads to active resistance often involving a hypersensitive response (HR) (Schell and Parker, 1990; Lamb, 1994).

This review focuses on fungal *Avr* genes. Recent results on *Avr* gene sequences, *Avr* gene expression, structure and activity of *Avr* gene products, and evolution of *Avr* genes are presented. The intrinsic biological function of *Avr* genes for the pathogen, the mechanisms of perception of AVR factors by plants carrying the matching *R* genes, and the exploitation of *Avr* and matching *R* genes in molecular resistance breeding will be discussed. Fungal elicitors that do not induce plant defense responses in a cultivar- or species-specific manner (for review see Ebel and Scheel, 1997), in addition to plant defense responses triggered after perception of *Avr* gene-encoded elicitors (Vera-Estrella *et al.*, 1994; Hammond-Kosack and Jones, 1996) will not be discussed here.

TABLE 1

The Gene-for-Gene Hypothesis Illustrated for Two Matching *Avr/R* Gene Pairs

Pathogen genotype	Host genotype	
	R1– r2r2	*r1r1 R2–*
Haploid		
A1 a2	Incompatible	Compatible
a1 A2	Compatible	Incompatible
Diploid/dikaryotic		
A1– a2a2	Incompatible	Compatible
a1a1 A2–	Compatible	Incompatible

STRUCTURE AND PROPERTIES OF *Avr* GENES AND THEIR PRODUCTS

By definition *Avr* genes restrict the host range of plant pathogenic fungi. Therefore, *Avr* genes have received

TABLE 2
Currently Cloned Fungal Avirulence Genes

Pathogen	Avr gene	Specificity	Homology	References
Cladosporium fulvum	Avr9	Tomato/Cf-9 genotypes	Cystine-knot peptide (structural)	Van den Ackerveken *et al.* (1992)
				Vervoort *et al.* (1997)
	Avr4	Tomato/Cf-4 genotypes	None	Joosten *et al.* (1994)
	Ecp2	Tomato/Cf-ECP2 genotypes	None	Laugé *et al.* (1998)
Rhynchosporium secalis	nip1	Barley/Rrs1 genotypes	Toxin/Hydrophobin?	Rohe *et al.* (1995)
				Wevelsiep *et al.* (1993)
Magnaporthe grisea	AVR2-YAMO	Rice/Yashiro-mochi cultivar	Neutral Zn^{2+} protease (sequence motif)	Valent (1997)
	PWL2, PWL1	Weeping lovegrass	None	Sweigard *et al.* (1995)
				Kang *et al.* (1995)
Phytophthora parasitica	para1	*Nicotiana tabacum?*	None	Ricci *et al.* (1992)
				Kamoun *et al.* (1994)
Phytophthora infestans	inf1	*Nicotiana benthamiana*	None	Kamoun *et al.* (1998)

considerable attention from molecular biologists studying the interactions between fungi and plants. Consequently, in the past decade, much research has been focused on cloning and characterizing fungal *Avr* genes. To date eight *Avr* genes have been isolated (Table 2). Five *Avr* genes (*Avr9, Avr4, Ecp2, nip1*, and *AVR2-YAMO*) govern cultivar specificity fitting the original gene-for-gene hypothesis as proposed by Flor for the *M. lini*–flax interaction (Flor, 1946), while three *Avr* genes (*PWL1, PWL2*, and *inf1*) govern species specificity in fungus–plant interactions.

Avr GENES WITH CULTIVAR-SPECIFICITY

Avr Genes Avr 9, Avr 4, and Ecp 2 of Cladosporium fulvum

Cladosporium fulvum causes leaf mold on tomato and interacts with its host in a gene-for-gene manner (Figs. 1A, 1B, and 1C) (De Wit, 1992). Several monogenic dominant genes that confer resistance against *C. fulvum* (*Cf*–) have been identified in wild relatives of tomato and were introgressed into commercial cultivars. The presence of *Avr* genes *sensu stricto* Flor (1946) in *C. fulvum* could not be genetically demonstrated as the sexual form of this fungus is unknown. Histological studies on incompatible interactions involving various *Cf* genes showed that they are mainly of the HR type (Lazarovits and Higgins, 1976; De Wit, 1977). *C. fulvum* does not produce specialized feeding structures during colonization of tomato leaves and fungal growth remains confined to the apoplast (Lazarovits

and Higgins, 1976; De Wit, 1977). All molecules involved in communication between the fungus and its host plant are therefore present in apoplastic fluids (AFs) isolated from *C. fulvum*-infected plants, including the putative AVR molecules that act as specific elicitors on plants carrying the matching *R* genes.

The putative *Avr9* avirulence gene of *C. fulvum*, matching the *Cf-9* resistance gene in tomato, was the first *Avr* gene to be investigated in the *C. fulvum*–tomato interaction. AFs were prepared from susceptible plants inoculated with strains that cannot overcome the *Cf-9* gene and from races that can overcome the *Cf-9* gene. Upon injection of these AFs into leaves of *Cf-9*-containing and *Cf-9*-lacking plants, it was confirmed that a protein elicited specific HR in the injected area of *Cf-9*-containing plants (Table 3). This proteinaceous elicitor is present in AFs from all interactions involving races that cannot overcome the *Cf-9* gene, while it is absent in AFs from races that can overcome the *Cf-9* gene (Fig. 1D) (Scholtens-Toma *et al.*, 1989). Therefore, this elicitor was proposed to be the product (AVR9) of the putative *Avr9* gene of *C. fulvum*. The AVR9 elicitor has been purified (Figs. 1E and 1F) and was found to be a 28-amino-acid (aa) peptide (Scholtens-Toma and de Wit, 1988). The *Avr9* structural gene has been subsequently cloned (Van den Ackerveken *et al.*, 1992). It encodes a preprotein of 63 aa with a characteristic signal peptide for extracellular targeting. The 40-aa secreted peptide is further processed by endogenous and plant proteases into the mature 28-aa AVR9 elicitor (Van den Ackerveken *et al.*, 1993). Transfer of the *Avr9* gene into a wild-type *Avr9*⁻ strain is sufficient to render the resulting *Avr9*⁺ transgenic strain avirulent on *Cf-9*-containing plants (Van den Ackerveken *et al.*, 1992).
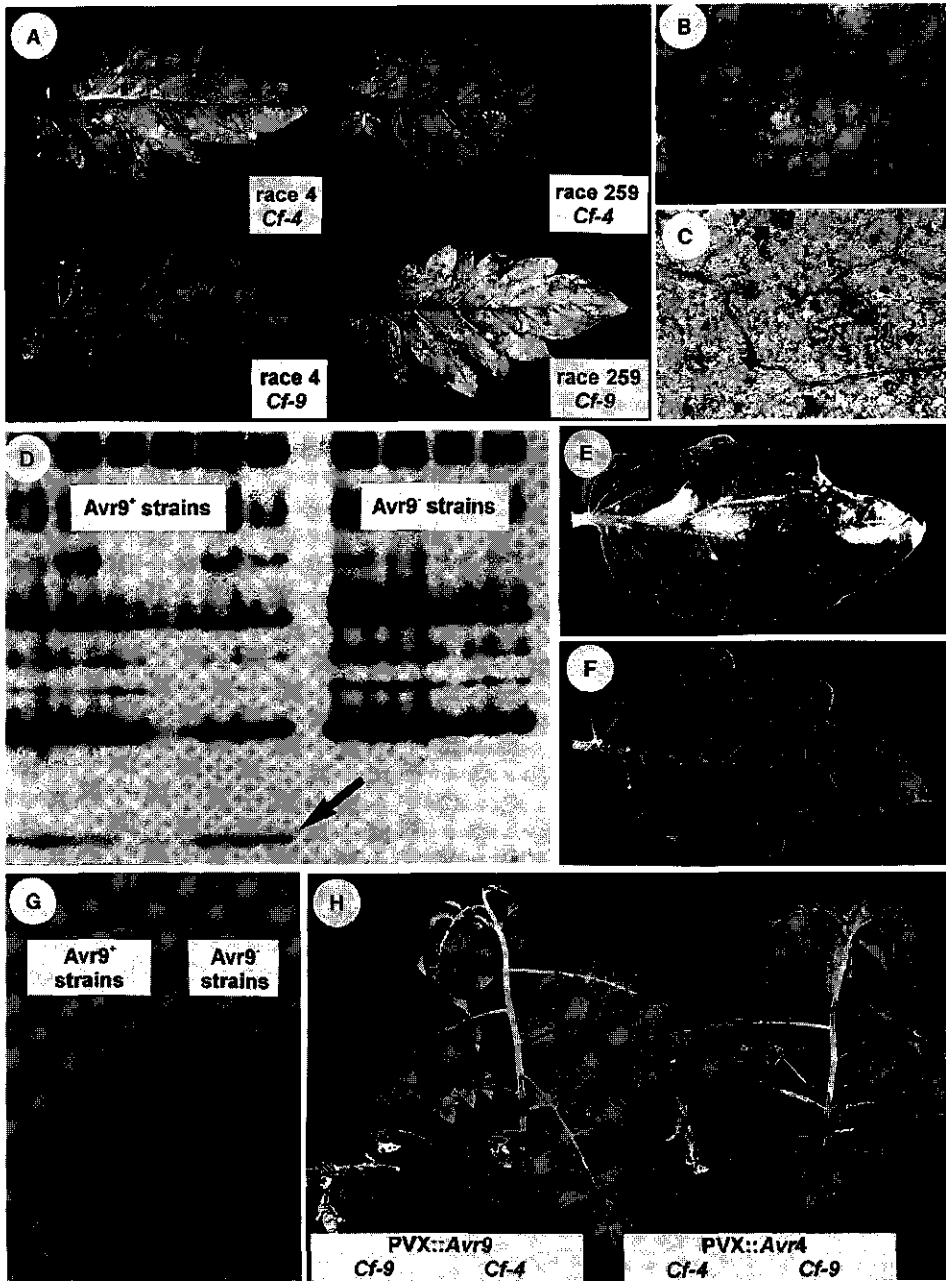
race 4
Cf-4

race 259
Cf-4

race 4
Cf-9

race 259
Cf-9

Avr9⁺ strains

Avr9⁻ strains

Avr9⁺
strains

Avr9⁻
strains

PVX::*Avr9*
Cf-9   *Cf-4*

PVX::*Avr4*
Cf-4   *Cf-9*

TABLE 3

HR-Inducing Activity of Elicitors from Various *Cladosporium fulvum*
Strains on Different Tomato Genotypes

C. fulvum strains	Elicitor(s) produced	Tomato genotypes			
		Cf-0	Cf-4	Cf-9	Cf-ECP2[a]
Wild-type races					
Race 5	AVR4, AVR9, ECP2	—[b]	HR	HR	HR
Race 4	AVR9, ECP2	—	—	HR	HR
Race 2.4.5.9	ECP2	—	—	—	HR
Mutant races					
Race 5-ΔAvr9[c]	AVR4, ECP2	—	HR	—	HR
Race 5-ΔEcp2[d]	AVR4, AVR9	—	HR	HR	—

[a] Laugé *et al.* (1998).

[b] —, no HR, the plant is susceptible.

[c] Marmeisse *et al.* (1993).

[d] Marmeisse *et al.* (1994).

Transformation-mediated disruption of *Avr9* in two wild-type *Avr9*+ strains changes the strains from avirulent to virulent on *Cf-9* plants (Marmeisse *et al.*, 1993). These results prove that the *Avr9* gene is the *Avr* gene matching the tomato *Cf-9* resistance gene. Expression studies have shown that the *Avr9* gene is strongly induced during infection (Fig. 1B). Furthermore, the *Avr9* gene is induced *in vitro* by nitrogen starvation (Van den Ackerveken *et al.*, 1994). In agreement with this finding, many (TA)GATA regulatory sequences described as targets for the *Aspergillus nidulans* AREA and *Neurospora crassa* NIT2 transcription factors involved in nitrogen metabolism are present in the promoter of *Avr9*. Current studies aim to evaluate the role of nitrogen in *Avr9* expression during the colonization of the host plant. No sequence variation has been detected among wild-type *Avr9*+ strains tested so far, and all wild-type strains known to overcome the *Cf-9* resistance

gene have been demonstrated by Southern blot analysis to lack the entire *Avr9* ORF (Fig. 1G) (Van Kan *et al.*, 1991). Thus wild-type *Avr9*− strains avoid recognition in *Cf-9* plants by not producing the AVR9 elicitor (Fig. 1D).

The *Avr4* gene that matches the tomato *Cf-4* resistance gene has been isolated in a way similar to the *Avr9* gene (Table 3) (Joosten *et al.*, 1994). The *Avr4* gene encodes a 135-aa preproprotein with a characteristic signal peptide for extracellular targeting. Like AVR9, the AVR4 product is further processed into a mature elicitor protein of 86 aa by plant and/or fungal proteases. Transformation of a wild-type *Avr4*− strain with the *Avr4* gene renders the transgenic strain avirulent on *Cf-4*-containing plants. *Avr4* gene expression is strongly induced during infection (Fig. 1C); however, specific conditions that induce *Avr4* gene expression *in vitro* have not been found so far. The sequence of the *Avr4* gene in various wild-type *Avr4*+ strains shows no polymorphism. All *avr4* alleles from wild-type *Avr4*− strains exhibit various single-point mutations, mainly single nucleotide substitutions resulting in the exchange of one amino acid in the AVR4 elicitor. In contrast to *Avr9*, all wild-type strains which are virulent on *Cf-4* plants contain *avr4* alleles that are strongly expressed during infection. However, none of the proteins encoded by the *avr4* alleles could be detected in the AFs of infected plants. Overexpression of the *avr4* alleles using the potato virus X expression vector (Chapman *et al.*, 1992) (Fig. 1H) showed that some *avr4* alleles encode a protein that is still recognized by *Cf-4* plants (Joosten *et al.*, 1997). Thus, different from wild-type *Avr9*− strains, wild-type *Avr4*− strains avoid recognition on plants containing the *Cf-4* gene by encoding unstable and/or mutated homologues of the AVR4 elicitor.

The *Avr4* and *Avr9* genes do not share homology with each other or with sequences present in databases. These

FIG. 1. (A) Leaves of *Cf-4* and *Cf-9* tomato genotypes inoculated with race 4 and race 2.5.9, resulting in a reciprocal gene-for-gene check; *Cf-4*/race 4 and *Cf-9*/race 2.5.9 give a compatible interaction while *Cf-4*/race 2.5.9 and *Cf-9*/race 4 give an incompatible interaction. Note the abundant brownish sporulating mycelium at the lower side of the leaf in the two compatible interactions. (B and C) Micrographs of tomato leaves inoculated with transgenic fungal strains containing promoter–GUS constructs. (B) *Avr9*–promoter–GUS transgenic strain inoculated on a susceptible plant; the interaction is compatible and strong staining develops around the vascular tissue. (C) *Avr4*–promoter–GUS transgenic strain inoculated on a resistant plant; the interaction is incompatible and limited staining occurs at the penetration sites (arrows) of the resistant plant. (D) Protein profiles of apoplastic fluids isolated from susceptible plants inoculated with six different *Avr9*+ strains of *C. fulvum* (note the presence of the AVR9 elicitor indicated by the arrow) and four different *Avr9*− strains (note the absence of the AVR9 elicitor). (E and F) Tomato leaflets injected with elicitor of *C. fulvum*. (E) Leaflet of *Cf-9* genotype injected with purified AVR9 elicitor; HR visualized as necrosis occurs in the injected area. (F) Leaflet of *Cf-0* genotype injected with purified AVR9 elicitor; no necrosis occurs. (G) Southern blot of three *Avr9*+ strains and two *Avr9*− strains probed with *Avr9* cDNA; note the presence of an *Avr9* signal in *Avr9*+ strains and the absence of signal in *Avr9*− strains. (H) Symptoms that have developed on *Cf-4* and *Cf-9* genotypes after inoculation with recombinant PVX viruses containing the cDNA of either the *Avr9* or the *Avr4* gene; note that for matching *R/Avr* gene pairs strong necrosis has developed following systemic viral spread.

two avirulence genes have in common that they encode low-molecular-weight cysteine-rich proteins that are secreted by *C. fulvum* during infection of tomato. Many additional low-molecular-weight proteins are present in AFs isolated from tomato leaves infected by *C. fulvum*. These additional proteins have been named ECPs, for extracellular proteins, and have been proposed to act as avirulence factors on yet unidentified resistant tomato genotypes. The two proteins, ECP1 (Joosten and de Wit, 1988) and ECP2 (Wubben *et al.*, 1994), have been assayed for HR-inducing activity on a collection of tomato breeding lines prescreened for resistance against *C. fulvum*. While none of these tomato lines display HR upon infiltration of purified ECP1, a few lines display HR upon infiltration of purified ECP2 (Table 3). In addition, it has been demonstrated that the ECP2-encoding gene acts as an *Avr* gene of *C. fulvum* on these lines that contain a monogenic resistance against *C. fulvum* operating through recognition of ECP2 (Laugé *et al.*, 1998). This indicates that avirulence of *C. fulvum* operates through recognition of its excreted proteins by different tomato genotypes, after which a HR is induced.

Avr Gene *nip1* of *Rhynchosporium secalis*

Rhynchosporium secalis causes leaf scald on barley. This fungus is assumed to interact in a gene-for-gene manner with its host, although, as for *C. fulvum*, lack of a sexual cycle of *R. secalis* prevents the genetic demonstration of *Avr* genes. After penetration of the cuticle, fungal mycelium remains subcuticular, and fungal growth is arrested after collapse of a few epidermal cells in incompatible interactions. Resistance of barley cultivars to *R. secalis* is assumed to result from early and strong induction of plant defense responses after perception of the fungal avirulence molecules.

Three low-molecular-weight necrosis-inducing peptides, NIP1, NIP2, and NIP3, have been purified from culture filtrate of the fungus grown *in vitro* and have been reported to act as aspecific toxins on barley, other cereals, and bean (Wevelsiep *et al.*, 1991, 1993). However, NIP1 also triggers the expression of two barley genes that encode pathogenesis-related (PR) proteins in a cultivar-specific manner. This elicitation of PR protein genes occurs specifically in barley cultivars that contain the *Rrs1* gene (resistance to *R. secalis* 1) and not in barley cultivars that do not contain *Rrs1*. The two mRNAs accumulate with similar timing when *Rrs1* barley cultivars are either treated with

purified NIP1 or inoculated with strains of *R. secalis* that are avirulent on *Rrs1* barley cultivars (Hahn *et al.*, 1993). The *nip1* gene of *R. secalis* that encodes NIP1 has been obtained via reverse genetics and has been proposed to represent the putative avirulence gene matching *Rrs1*, the *AvrRrs1* gene. Transfer of *nip1* to a wild-type *AvrRsr1*⁻ strain gave transformants that are avirulent on *Rrs1* barley cultivars, which proves that *nip1* and *AvrRsr1* are the same gene (Rohe *et al.*, 1995). Two *nip1* sequences encoding NIP1 proteins differing from each other by three amino acids are found among wild-type strains of *R. secalis* that are avirulent on *Rrs1* barley cultivars. Strains of *R. secalis* that are virulent on *Rrs1* barley cultivars have additional single-point mutations or have lost the *nip1* sequence completely.

Avr Gene *AVR2-YAMO* of *Magnaporthe grisea*

Magnaporthe grisea is a foliar pathogen on more than 50 different grass species, including rice, on which it causes blast disease. Since the sexual stage of *M. grisea* is controlled under laboratory conditions (Herbert, 1971), the genetics of virulence and avirulence have been studied extensively.

Crosses between strains of *M. grisea* that are differentially pathogenic on various rice cultivars have revealed the existence of many gene-for-gene relationships with cultivar specificity between the fungus and rice (Valent *et al.*, 1991; Silué *et al.*, 1992). The avirulence gene *AVR2-YAMO*, which prevents infection of the Yashiro-mochi cultivar, has been isolated by positional cloning (Valent, 1997). It encodes a 223-aa protein with a putative signal peptide. The sequence of the *AVR2-YAMO* gene does not share significant homology with other known proteins, except for a small domain which shares homology with the active site of neutral Zn^{2+} proteases. Since the gene is located at the tip of chromosome 1, many spontaneous virulent mutants result from DNA deletions or insertions. Virulent isolates carrying point mutations in the putative active site of neutral Zn^{2+} proteases have also been found.

Avr GENES WITH SPECIES-SPECIFICITY

Avr Genes *PWL1* and *PWL2* of *M. grisea*

Despite a wide host range, individual isolates of *M. grisea* are often restricted to one or a few plant species.

The existence of *Avr* genes governing species specificity has been demonstrated (Valent, 1997). Crosses between strains of *M. grisea* that either could or could not infect weeping lovegrass (*Eragrostis curvula*) have led to the identification of two *PWL* genes (govern pathogenicity toward weeping lovegrass) that showed characteristics of *Avr* genes in preventing *M. grisea* from causing disease on this plant species. *PWL1* originates from a strain virulent on finger millet (*Eleusine coracana*), while *PWL2* has been identified in a strain virulent on rice. The *PWL2* avirulence gene has been isolated by positional cloning (Sweigard *et al.*, 1995). Transformation of a wild-type *PWL2⁻* strain with the *PWL2* gene renders the strain avirulent on weeping lovegrass. The encoded product is predicted to be a hydrophilic protein of 145 aa with a putative signal peptide. A search for *PWL2* homologues in various *M. grisea* isolates has shown the *PWL* genes to be members of a small gene family. *M. grisea* isolates that are virulent on weeping lovegrass contain *PWL2* homologues (Kang *et al.*, 1995). *PWL1* has been cloned by homology to *PWL2*. Interestingly, although the *PWL1* gene shares only 75% aa identity with *PWL2*, it is still a functional homologue of *PWL2*, as it prevents infection of weeping lovegrass. In contrast, another *PWL* homologue, *PWL3* (51% aa identity with *PWL2*), from the finger millet isolate is nonfunctional, as it does not prevent infection of weeping lovegrass. A fourth *PWL* homologue, *PWL4*, has been isolated from a weeping lovegrass isolate. The latter is more related to *PWL3* (72% aa identity) than to *PWL2* (57% aa identity) and, like *PWL3*, it does not confer avirulence on weeping lovegrass. Surprisingly, when the open reading frame (ORF) of *PWL4* is placed under the control of either the *PWL1* or the *PWL2* promoter, it confers avirulence on weeping lovegrass, while similar constructions with the *PWL3* ORF do not. This suggests that *PWL4* is nonfunctional due to a defective promoter, while *PWL3* is nonfunctional due to mutations within its ORF (Kang *et al.*, 1995).

The Genes Encoding Elicitins of Phytophthora spp.

For a long time the involvement of the elicitins as avirulence determinants at the species level has been proposed (Ricci *et al.*, 1992; Kamoun *et al.*, 1994). This family of low-molecular-weight proteins from species of *Phytophthora* and *Pythium* (Kamoun *et al.*, 1997) induces specific necrosis in all *Nicotiana* species tested so far. The main reason for considering these proteins as avirulence determinants on *Nicotiana* species came from studies on *Phytophthora parasitica*. The level of elicitin production

has been analyzed for isolates of *P. parasitica* obtained from tobacco and other host plants. A strong negative correlation was found between virulence on tobacco and the level of elicitin production (Ricci *et al.*, 1992; Bonnet *et al.*, 1994). Consequently, the *para1* gene encoding the elicitin parasiticein of *P. parasitica* has been proposed to act as a species-specific *Avr* gene (Kamoun *et al.*, 1993). Lack of an efficient genetic transformation system and the diploid nature of this fungus have prevented so far confirmation of the role of *para1* in species specificity of *P. parasitica*. Recent studies on the elicitin infestin encoded by the *inf1* gene of the closely related oomycete *Phytophthora infestans* give molecular support for the role of elicitins in species specificity. Wild-type strains of *P. infestans* are avirulent on *Nicotiana* species and are arrested at an early stage of infection on these plants. *inf1⁻* strains of *P. infestans*, obtained through silencing of *inf1* in wild-type *inf1⁺* strains, can complete the infection cycle up to sporulation on *Nicotiana benthamiana* (Kamoun *et al.*, 1998). Therefore, at least for this interaction, the *inf1* gene appears to act as an *Avr* gene with species specificity in preventing infection of *N. benthamiana* by *P. infestans*.

Avr Genes That Are Currently Being Cloned

The cloning of additional fungal *Avr* genes is in progress. These include *Avr* genes such as *AvrCo39*, *AVR1-MARA*, *AvrIrat7-1*, *AvrMednoï-1*, and *AvrKu86-1* of *M. grisea* giving cultivar specificity on rice (Leong *et al.*, 1994; Mandel *et al.*, 1997; Dioh *et al.*, 1996) and *AvrLm1* of *Leptosphaeria maculans* giving cultivar-specificity on canola (Ansan-Melayah *et al.*, 1995). *Avr* genes from *Phytophthora sojae*, *P. infestans*, and *M. lini* giving cultivar specificity on soybean, potato and flax, respectively, are currently being mapped (Whisson *et al.*, 1994, 1995; Van der Lee *et al.*, 1997; Timmis *et al.*, 1990). Reverse genetics is applied to clone the genes encoding two recently reported cultivar-specific elicitors of *Uromyces vignae* acting on cowpea (D'Silva and Heath, 1997).

INTRINSIC FUNCTION OF Avr GENES

Scientists have always been intrigued by the presence of *Avr* genes in the genome of many plant pathogens. The definition of a fungal *Avr* gene concerns the inability of a

particular fungal strain to cause disease on a particular plant species or cultivar. The definition does not imply an intrinsic function of the *Avr* gene for the fungus itself. As *Avr* genes restrict the host range of pathogens, one would expect these genes to disappear quickly through selection pressure exerted by a resistant plant. Therefore, an intrinsic pleiotropic function(s) for the fungus itself has been hypothesized in order to explain the maintenance of *Avr* genes in a population of fungal pathogens (Knogge, 1996). *Avr* genes could have either housekeeping or pathogenicity functions, encoding products such as enzymes involved in the degradation of plant substrates, toxins, or suppressors of the host defense responses. Examination of phenotypes of wild-type *Avr⁻* strains might give clues to a putative intrinsic function of a given *Avr* gene. However, in most cases only a few wild-type *Avr⁻* strains have been described. In addition, in nature *Avr⁻* strains may have evolved to compensate for the loss of the intrinsic *Avr* gene functions by complementation through a functional homologue(s). A reliable way to test the putative intrinsic function of an *Avr* gene is to compare, *in vitro* and/or *in planta*, the phenotype of an isogenic *Avr⁻* strain created by gene disruption with the phenotype of the corresponding near-isogenic wild-type *Avr⁺* parental strain.

Currently, little information is available concerning the putative functions of the *Avr* genes that have been described above. *Avr9* disruption mutants are not affected in their growth pattern. They do not display an altered phenotype *in vitro* compared with the isogenic wild-type parental strains, neither do they seem to be affected in their pathogenicity on tomato (Laugé *et al.*, 1998). Putative involvement of the *Avr9* gene in the nitrogen metabolism of *C. fulvum* has been proposed, as discussed before. Phenotypes of wild-type *Avr4⁻* strains are not different from those of *Avr4⁺* strains *in vitro* or *in planta* (M. H. A. J. Joosten, pers. comm.), but *Avr4* disruption mutants have yet to be created to confirm the lack of an important role of *Avr4* for *C. fulvum*. In contrast, *Ecp2* was originally characterized as a gene with an important role in pathogenicity of *C. fulvum*, as an *Ecp2* disruption mutant displayed reduced leaf colonization and reduced conidiation compared with the wild-type parental strain (Laugé *et al.*, 1997).

Wild-type *nip1⁻* strains as well as *nip1* disruption mutants of *R. secalis* have also been shown to exhibit a significant reduction in pathogenicity on barley (Rohe *et al.*, 1995; Knogge, 1996). The toxic activity of NIP1 that acts through stimulation of plant plasmalemma ATPase is likely to account for the pathogenicity function of NIP1. The spacing of cysteine residues in the NIP1 protein was found to be similar to that found in the family of the fungal hydrophobins, but no such function for NIP1 has been demonstrated yet.

A putative intrinsic function for *PWL1* and *PWL2* of *M. grisea* and *inf1* of *P. infestans* cannot be proven easily. These genes are members of gene families with potential functional homologues (Sweigard *et al.*, 1995; Kamoun *et al.*, 1997). Cumulative disruption of all genes separately and/or in combination will be required to assess the possible functions of *PWL1*, *PWL2*, and *inf1*.

AVIRULENCE PERCEPTION

The mechanisms of recognition of *Avr* gene products by plants carrying the matching resistance gene are still largely unknown. The simplest model predicts that the *Avr* gene product is the elicitor molecule which is directly perceived by the resistant plant via the action of an *R*-gene-encoded receptor (Keen, 1990; De Wit, 1992). Several research groups are trying to find experimental evidence to prove or disprove this model.

The *Avr* genes that have been obtained through the reverse genetics approach (*Avr4*, *Avr9*, *Ecp2*, *nip1*, and *inf1*) have been isolated based on a specific induction of defense responses (HR or induction of PR proteins) by their encoded products in plants carrying the matching *R* gene. These *Avr* genes encode the eliciting AVR product, which is perceived by the resistant plant directly or after processing. Some of the *Avr* genes that have been obtained through positional cloning might not directly encode the eliciting compound. The *PWL2* gene product is predicted to be extracellular. However, injection of weeping lovegrass with the purified recombinant PWL2 protein did not elicit any detectable plant response (Sweigard *et al.*, 1995). The *AVR2-YAMO* gene product, which has potential protease activity, might be responsible for the release of an active elicitor molecule. The latter has been described for the avirulence gene *avrD* of the bacterium *Pseudomonas syringae* pv. *tomato*, the product of which is responsible for the synthesis of syringolide elicitors (Keen *et al.*, 1990).

Biochemical studies have been initiated to unravel the molecular mechanisms of recognition of the HR-eliciting AVR9 protein by *Cf-9* plants. Binding studies using ¹²⁵I-labeled AVR9 showed the presence of a high-affinity binding site for the AVR9 protein in membrane fractions of

tomato. Surprisingly, this high-affinity binding site for AVR9 appeared to be present in both *Cf-9*-containing and *Cf-9*-lacking plants (Kooman-Gersmann *et al.*, 1996). Therefore this binding site could not represent the *Cf-9* gene product. Consequently, the biochemical model that predicts that the *Cf-9* resistance gene encodes a unique receptor for AVR9 perception has to be refined. Most probably the AVR9 elicitor binds to a coreceptor that is part of a receptor complex including the *R*-gene-encoded product (Kooman-Gersmann *et al.*, 1998). Similar studies are under way for the AVR4 and NIP1 elicitors (Joosten *et al.*, 1997; Knogge, 1996).

EXPLOITATION OF *Avr* GENES IN MOLECULAR RESISTANCE BREEDING

The interest in studying *Avr* genes is obvious for understanding mechanisms of plant resistance. However, as stated above, *Avr* genes exert their function in concert with their host counterpart, the matching *R* genes. Concomitantly with the growing interest in cloning *Avr* genes, *R* genes have also received much attention. Apart from fundamental interest in the gene pairs, engineering resistance by genetic transfer of *Avr/R* gene pairs to plants has become an object of study for biotechnological applications. This method has been introduced as the two-component sensor system (De Wit, 1992). The strategy consists of transferring an *Avr/R* gene pair to a given crop plant. By regulating both in time and in space the expression of the *Avr/R* gene pair, one can envisage artificial resistance of the transgenic crop as taking place against any pathogen which can be inhibited by HR. Along these lines, the *Avr9/Cf-9* gene pair is now being tested for suitability in molecular resistance breeding. Constructs have been made in which either the *Avr9* gene or the *Cf-9* gene (Jones *et al.*, 1994) is under control of a pathogen-inducible promoter, *Pgst1* (Strittmatter *et al.*, 1996). These constructs have been transferred to tomato plants (Fig. 2). Progenies obtained after selfing of primary transformants were subsequently inoculated with a wild-type *Avr9⁻* strain of *C. fulvum*. Several transgenic lines showed resistance to this strain, with HR induction at the site of infection, thus pointing to transient induction of the *Avr9* transgene. As a result of the HR, fungal growth is arrested and the plant becomes resistant (De Wit, 1997). Use of the *Avr9/Cf-9* gene pair only in a homologous system like the
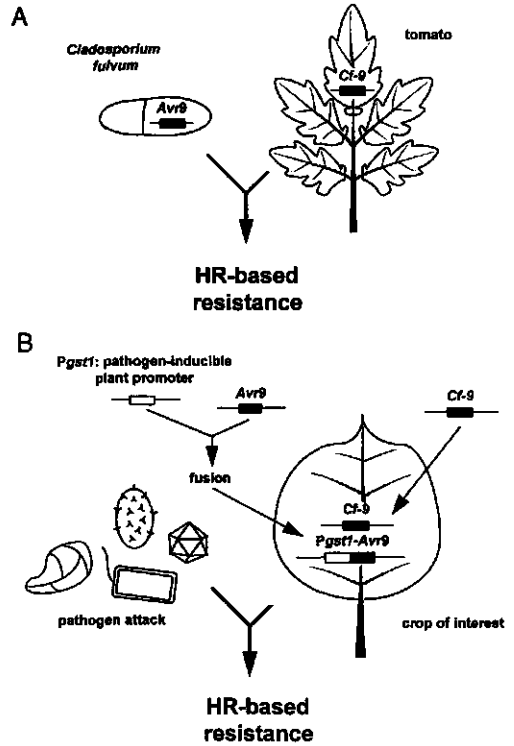

FIG. 2. (A) Natural resistance: The gene-for-gene system. The *Avr9/ Cf-9* gene pair governs natural HR-based resistance in the *C. fulvum–*tomato interaction. (B) Engineered resistance: The two-component system. Upon transfer of *Cf-9* and *Avr9*, which is placed under control of the pathogen-inducible promoter *Pgst1*, to a recipient crop; engineered HR-based resistance occurs against pathogens that can infect this plant.

C. fulvum–tomato interaction would give only limited application. Fungi have various modes of infection, and similarly plants may have various modes of defense responses. Signal transduction pathways involved in defense of plants may vary from one family to another or even from one genus to another. Nevertheless, HR seems to be a common resistance mechanism in many plants. Experiments involving the transfer of the *Avr9/Cf-9* gene pair to solanaceous plants other than tomato, to nonsolanaceous plants such as *Arabidopsis*, and to monocots such as rice are presently in progress. Resulting transgenic plants are currently being tested for resistance toward their pathogens.

OUTLOOK

Our molecular genetic knowledge of fungal *Avr* genes is presently based on the analysis of only eight genes that have little in common. They share sequence homologies neither at the nucleotide level nor at the amino acid level. Some are only expressed *in planta* while others are expressed *in vitro* as well as *in planta*. However, they are all known or predicted to encode extracellular proteins. The plant *R* genes *Cf-4* (Thomas *et al.*, 1997) and *Cf-9* (Jones *et al.*, 1994) acting against *C. fulvum* are the only *R* genes directed against a fungus for which the matching *Avr* genes, *Avr4* and *Avr9*, respectively, have been cloned. *Cf-4* and *Cf-9* encode predicted extracytoplasmic glycoproteins with a short intracellular anchor which would fit a model for extracellular perception of the AVR4 and AVR9 elicitors excreted by the fungus in the apoplast. Most plant pathogenic fungi develop intimate contact with the host plasma membranes through structures such as haustoria that are required for uptake of nutrients (Agrios, 1997). The other cloned *R* genes conferring resistance against fungi; *L6* (Lawrence *et al.*, 1995) and *M* (Anderson *et al.*, 1997), directed against *M. lini; RPP5* (Parker *et al.*, 1997), directed against *Peronospora parasitica;* and *I2* (Ori et al, 1997), directed against *Fusarium oxysporum* f. sp. *lycopersici*, appear to encode intracellular proteins. The matching *Avr* genes have not been characterized yet; however, the putative cytoplasmic localization of the products of these *R* genes suggests that fungal elicitors may actively enter plant cells through the haustorial membrane. Such a possibility is illustrated by plant pathogenic bacteria. Most bacterial *Avr* gene products are cytoplasmic and are subsequently injected actively into the cytoplasm of the host cell through a type III secretion system (Alfano and Collmer, 1996; Leach and White, 1996). Accordingly, most of the bacterial *R* genes that have been cloned encode cytoplasmic proteins (De Wit, 1997). Extracellular perception of elicitors such as AVR4 and AVR9 of *C. fulvum* may eventually be an exception rather than a rule. However, it is too early to draw conclusions on the mode of perception of fungal avirulence factors by plants since only few have been characterized in detail and no direct interaction between a fungal avirulence factor and an *R* gene product has been demonstrated unequivocally.

Avr gene products detected by the surveillance system of the plant must embody important pleiotropic functions; otherwise the encoding genes would have been lost from the pathogen population quickly. If an *Avr* gene does not have a clear direct effect on pathogenicity while colonizing the host plant, it may still play an important role in spore dispersal or survival (overwintering or bridging periods when there are no host plants available). These latter features are difficult to qualify or quantify under laboratory conditions. So far, five of the cloned bacterial *Avr* genes, *avrA, avrB, avrE, avrRPM1*, and *avrBs2* (Alfano and Collmer, 1996; Leach and White, 1996), and two fungal *Avr* genes, *Ecp2* and *nip1*, have been shown to encode proteins that function as factors of pathogenicity. This demonstrates that plants have evolved monitoring systems to recognize pathogenicity factors of pathogens, which turn them into elicitors of HR-based resistance. Although most pathogen *Avr* genes cloned so far show no or little homology to each other, one would expect that *Avr* genes that encode important pathogenicity factors could have homologous counterparts in closely or even distantly related pathogens. The matching *R* genes would be potentially durable genes as the pathogen should not lose or adapt important pathogenicity factors easily.

The homology in sequence and presumably also in the structure of the proteins encoded by plant *R* genes targeted against viruses, bacteria, fungi, and nematodes, that have been cloned from different plant species such as *Arabidopsis*, flax, rice, tobacco, and tomato, is intriguing (Jones and Jones, 1996; De Wit, 1997). It is hypothesized that *R* genes, in addition to being part of the surveillance system, might embody additional functions for plants. They might act as receptors for yet unidentified endogenous ligands and might be involved in physiological and/or developmental regulation. The *R* gene homology also suggests that their products represent versatile (co)receptor molecules for binding ligands as monomers, dimers, or heteromers as has been reported in mammals for receptors of growth factors (Heldin, 1995).

CONCLUSION

The fine tuning between pathogen *Avr* genes and matching plant *R* genes seems to be the outcome of coevolution between a pathogen and its host over a long time. In natural ecosystems plants will generate new recognition specificities targeted against deleterious pathogens. Simultaneously, the pathogen will develop strains that overcome the resulting resistances. The accumulation of surveillance specificities is expected to have its cost for the plant. In a similar way, loss or mutational adaptation of an *Avr* gene which has a crucial intrinsic function has its cost for the pathogen. It is probably the balance between

the advantage of having the intrinsic function(s) and the drawback of having a restricted host range that decides whether a particular *Avr* gene is kept or eliminated. Thus some avirulence genes will occur only briefly in the populations of fungal pathogens, while others could persist for a longer time. What we currently observe is presumably only a snapshot of a very dynamic process in the interactions between pathogenic fungi and their host plants.

ACKNOWLEDGMENTS

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Chapter 3

The *in planta*-produced extracellular proteins ECP1 and ECP2 of *Cladosporium fulvum* are virulence factors

The In Planta–Produced Extracellular Proteins ECP1 and ECP2 of *Cladosporium fulvum* Are Virulence Factors

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The two extracellular proteins ECP1 and ECP2 are abundantly secreted by the plant-pathogenic fungus *Cladosporium fulvum* during colonization of the intercellular space of tomato leaves. We examined the involvement of both proteins in pathogenicity and virulence of this fungus. ECP1-deficient, ECP2-deficient, and ECP1/ECP2-deficient isogenic *C. fulvum* strains were created by targeted gene replacement. Upon inoculation onto susceptible 6-week-old tomato plants, all three mutants showed reduced virulence. Deficiency in ECP2 resulted in a strain that poorly colonized the leaf tissue and secreted lower amounts of the in planta–produced ECP3, AVR4, and AVR9 proteins than the wild-type strain. The ECP2-deficient strain produced little emerging mycelium and few conidia. Deficiency in ECP1 did not significantly modify colonization of the leaf tissue, but reduced secretion of in planta–produced proteins. The ECP1-deficient strain emerged from stomata of the lower epidermis, but failed to sporulate as abundantly as the wild-type strain. A strain deficient in both ECP1 and ECP2 proteins had a phenotype similar to that of the ECP2-deficient strain. Accumulation of pathogenesis-related proteins and induction of late responses, such as leaf desiccation and abscission, occurred more quickly and more severely in tomato after inoculation with the ECP1-, ECP2-, and ECP1/ECP2-deficient strains than after inoculation with the wild-type strain. Moreover, partial collapse of stomatal guard cells occurred at emergence of the ECP2-deficient strain. These results indicate that the ECP1 and ECP2 proteins play a role in virulence of *C. fulvum* on tomato and suggest that both are involved in suppression of host defense responses.

The fungal pathogen *Cladosporium fulvum* is responsible for the leaf mold disease of tomato. During the last decade, this plant-pathogen interaction has been studied in great detail and is used as a gene-for-gene model system (Oliver 1992; De

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Wit 1995). Molecular studies have resulted in the cloning of the fungal avirulence genes *Avr9* (Van den Ackerveken et al. 1992) and *Avr4* (Joosten et al. 1994), and the cloning of the tomato resistance genes *Cf-9* (Jones et al. 1994) and *Cf-2* (Dixon et al. 1996).

Little is known about the pathogenicity and virulence factors of this fungus that are responsible for its ability to colonize the apoplastic space of tomato leaves. Biochemical studies in our laboratory have shown that mannitol concentration increases drastically in the extracellular space during colonization of susceptible tomato leaves by *C. fulvum* (Joosten et al. 1990). Mannitol dehydrogenase, which could be involved in the nutrition of the fungus inside the leaf tissue and in pathogenesis, is currently under investigation (Noeldner et al. 1994). Mutagenesis experiments resulted in a large set of *C. fulvum* mutants that were affected in their virulence, but little information on the observed phenotypes was available (Kenyon et al. 1993).

As growth of the fungus remains restricted to the apoplastic space surrounding mesophyll cells during its whole life cycle (De Wit 1977; Lazarovits and Higgins 1976), molecules involved in communication between the fungus and the plant have been identified by analysis of apoplastic washing fluids (AF) from infected leaves (De Wit et al. 1986). This approach allowed the isolation of the two proteinaceous elicitors, AVR9 (Scholtens-Toma and De Wit 1988) and AVR4 (Joosten et al. 1994). In addition, many other low-molecular-weight proteins are present in AF, several of which are of fungal origin (De Wit et al. 1986). Two of these fungal proteins, named ECP1 and ECP2 for extracellular proteins 1 and 2, are present in abundance in AF of infected tomato leaves and have been purified (Joosten and De Wit 1988; Wubben et al. 1994). The two encoding genes, *Ecp1* and *Ecp2*, have been cloned (Van den Ackerveken et al. 1993). Northern (RNA) hybridization analyses performed on *C. fulvum* grown in vitro and in planta showed that the expression of both genes is highly induced during pathogenesis. Subsequent β-glucuronidase reporter studies revealed that there is expression only in the fungal mycelium growing in the apoplastic space (Wubben et al. 1994). Immunocytolocalization studies showed that, during pathogenesis, accumulation of both proteins occurs in the ex-

tracellular matrix between the fungal hyphae and the mesophyll cells (Wubben et al. 1994). These data suggest that the ECP1 and ECP2 proteins are good candidates for pathogenicity or virulence factors of *C. fulvum.*

A strain in which the *Ecp2* gene has been deleted is still able to cause disease on 2-week-old seedlings axenically grown, inoculated, and incubated in closed jars, indicating that ECP2 is dispensable for *C. fulvum* during pathogenesis (Marmeisse et al. 1994). We made an additional isogenic strain deficient for ECP1 and a strain deficient for both ECP1 and ECP2 by transformation-mediated gene replacement. Virulence of the three deficient strains was examined on 6-week-old susceptible plants in the glasshouse, rather than on 2-week-old seedlings in vitro, because these conditions better reflect the natural situation: *C. fulvum* is known to be a better leaf pathogen of adult plants than a pathogen of seedlings. In parallel, the plant defense responses induced by the ECP-deficient strains were investigated to get a better understanding of the biological functions of the ECP1 and ECP2 proteins during pathogenesis.

Here we report on a series of pathogenicity assays of mutant strains of *C. fulvum* that lack the *Ecp1* gene, the *Ecp2* gene, or both genes. The assays showed that deletion of either *Ecp1, Ecp2,* or both genes significantly decreases virulence of *C. fulvum* on 6-week-old tomato plants.

RESULTS

Generation of single and double *Ecp* gene replacement mutants.

A transformation plasmid carrying a selectable marker flanked by sequences upstream and downstream of the *Ecp1* start codon was constructed. Details of the construction steps are presented in Figure 1A. The final construct carries a truncated version of the *Ecp1* gene in which both the 1.6-kb proximal sequence of the promoter and the first 21 codons of the open reading frame are lacking. A double homologous recombination event of this DNA strand at the *Ecp1* locus results in replacement of the wild-type *Ecp1* sequence by a promoterless, untranslatable, truncated *Ecp1* gene as shown in Figure 1B. This construct was used for transformation of both the wild-type strain race 5 and the Δ*Ecp2*-D39 strain in which the *Ecp2* open reading frame had already been deleted (Marmeisse et al. 1994) to create *Ecp1* and *Ecp1/Ecp2* deletion mutants, respectively. Perfect gene replacement strains free of ectopic integrations of the transforming plasmid were identified either directly by Southern blot analysis on genomic DNA for the wild-type strain race 5, or by polymerase chain reaction (PCR) and subsequent Southern blot analysis for the Δ*Ecp2*-D39 strain. Two out of 100 transformants were found to lack the *Ecp1* locus, and one of these two showed an additional illegitimate recombination event. When the 865-bp *XhoI/HindIII* fragment from the replacement plasmid, which contains most of the *Ecp1* open reading frame and 3′ sequences, was used as a probe (Fig. 1C), the exact *Ecp1* replacement transformant, designated Δ*Ecp1*-77, exhibited a unique 1-kb *XhoI* replacement hybridization band and lacked the wild-type 7-kb *XhoI* band.

It was assumed that more transformants of the Δ*Ecp2*-D39 strain had to be screened to obtain a strain in which both *Ecp1* and *Ecp2* are replaced, as the promoter and terminator sequences used in the *Ecp1* replacement marker cassette were identical to those of the *Ecp2* gene replacement cassette. These sequences might therefore act as targets for homologous recombination at the *Ecp2*-replaced locus as well. Three hundred and fifty transformants were prescreened by PCR for absence of the wild-type *Ecp1* locus with the pair of primers indicated in Figure 1B. From the transformants that lacked the *Ecp1* locus, one double mutant, designated Δ*Ecp1*/Δ*Ecp2*-B94, did not show any ectopic integration (Fig. 1C).

Western blot (immunoblot) analysis of proteins present in AF isolated from tomato leaves inoculated with the Δ*Ecp1*-77 or the Δ*Ecp1*/Δ*Ecp2*-B94 strains confirmed that no ECP1 protein was produced by those strains (Fig. 1D) whereas ECP3, another fungal interaction-specific protein, was detected in both strains.

Characterization of ECP-deficient strains in vitro.

The ECP-deficient strains and the wild-type strain did not show differences in growth rate and morphology when grown either on potato dextrose agar (Fig. 2) or in B5 liquid medium (data not shown). No significant differences in sporulation rate and spore germination were observed between the mutants and the wild-type strain when grown on solid media. Southern analyses on DNA isolated from the mutant strains after several rounds of subculture did not show any changes in hybridization patterns, indicating that the *Ecp*-replaced strains are stable. Contour-clamped homogeneous electric field (CHEF) gel analysis of the mutant strains did not reveal changes in chromosome mobility when compared with the wild-type strain, suggesting that no significant chromosomal rearrangements had occurred (data not shown).

Pathogenicity assays with the ECP-deficient strains.

When assayed on 2-week-old tomato seedlings, the ECP1-deficient strain caused normal disease symptoms, indicating no requirement of *Ecp1* as a pathogenicity factor for *C. fulvum.* However, this assay, in which the fungus colonizes cotyledons and primary leaves of seedlings in vitro, may not represent the natural situation, in which *C. fulvum* infects expanded leaves of mature tomato plants. Infected seedlings become desiccated after infection, whereas the upper side of infected mature leaves becomes chlorotic when the fungus initiates sporulation on the lower side of the leaf. For this reason, the ECP-deficient strains were also tested on 6-week-old tomato plants. Six-week-old plants developed chlorotic symptoms about 2 weeks after inoculation, irrespective of whether they were inoculated with the wild-type, the ECP1-deficient, the ECP2-deficient, or the double ECP1/ECP2-deficient strains. The double-deficient strain, which was found to be as pathogenic as the ECP2-deficient strain, was not studied in further detail.

Increase in fungal biomass during colonization of tomato leaves was estimated by light microscopy (Fig. 3) and enzyme-linked immunosorbent assays (ELISAs) (Fig. 4). In ELISAs, accumulation of three in planta–produced fungal proteins, ECP3, AVR4, and AVR9, was followed in AF from infected leaves. The ECP1-deficient strain colonized the leaf tissue as efficiently as the wild-type strain (Fig. 3A and B), while it produced less of the three in planta, extracellular proteins when compared with the wild-type strain (Fig. 4). Thus, ECP1 deficiency does not appear to severely affect the ability
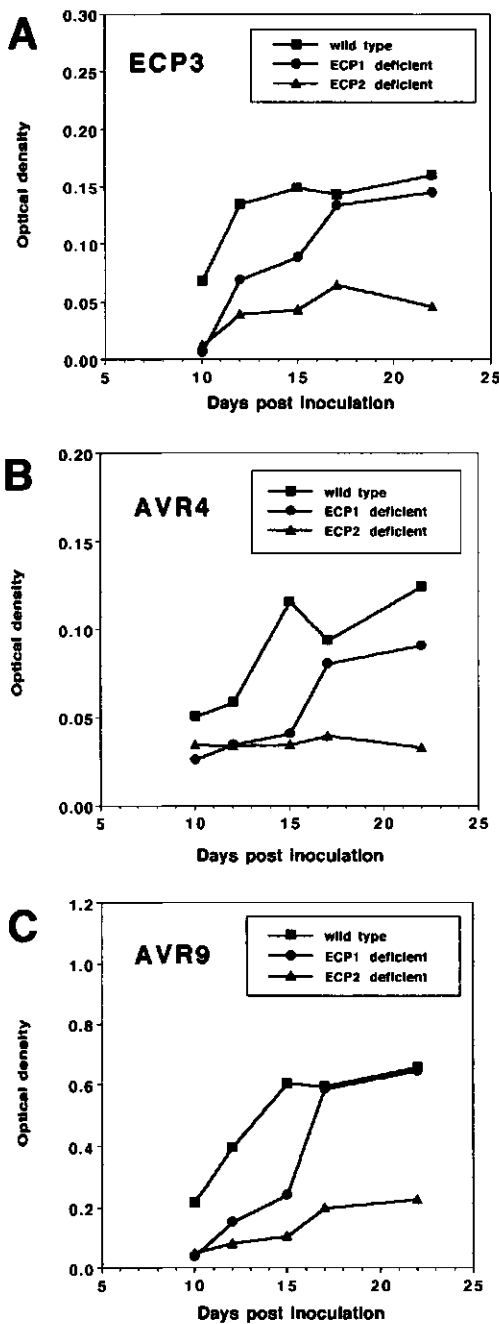
Fig. 4. Quantitative enzyme-linked immunosorbent assays on 20 μl of apoplastic washing fluid isolated from tomato leaves infected by the wild-type, ECP1-, and ECP2-deficient strains at several time points after inoculation, with **(A)** ECP3 antibodies, **(B)** AVR4 antibodies, and **(C)** AVR9 antibodies. Optical density was determined at 405 nm.

points (Fig. 7, lanes 6, 7, 8, and 9). Thus, lack of production of either ECP1 or ECP2 resulted in additional accumulation of PR proteins in tomato.

During later stages of infection (4 to 5 weeks post inoculation), it was observed that wilting and abscission of leaves were more pronounced for plants inoculated with either of the ECP-deficient strains than for plants inoculated with the wild-type strain (data not shown). Collapse of stomatal guard cells at the time of emergence of mycelium through stomata was frequently observed for plants inoculated with the ECP2-deficient strain (Fig. 8B). This feature was never observed in plants inoculated with the wild-type strain (Fig. 8A) or the ECP1-deficient strain (data not shown).

Virulence of the ECP-deficient strains on detached leaves.

In order to test whether the reduced virulence displayed by the ECP-deficient strains could be affected by the physiological conditions of host tissue, we did a two-step pathogenicity assay. Six-week-old plants were inoculated with conidia of the wild-type and the ECP-deficient strains. After 2 weeks, at the time when the wild-type strain was about to develop extrafoliar mycelium, infected leaflets were detached and placed at room temperature under 100% relative humidity in the dark. After incubation for one additional week, fungal development on the detached leaflets was compared with fungal development on the leaflets that had remained attached to the plant. On leaflets attached to the plant, the ECP-deficient strains remained affected in their development in comparison with the wild-type strain as described above. On the detached leaflets, all ECP-deficient strains started further development. The ECP1-deficient strain showed increased sporulation, and both the ECP2-deficient and the double ECP1/ECP2-deficient strains produced additional emerging mycelium (data not shown).

DISCUSSION

Gene replacement in *C. fulvum*.

Gene replacement was used to test the role of the two extracellular proteins ECP1 and ECP2 in pathogenicity and virulence of *C. fulvum*. The *Ecp1* locus is surrounded by repetitive DNA that hampered the design of a replacement construct. *Ecp1* replacement could theoretically have resulted in deletion of another gene in addition to *Ecp1*. However, the results of the co-inoculation experiments led us to conclude that the complementation observed is most probably due to ECP1, and that no other important function was deleted during *Ecp1* replacement. This is supported by three observations. First, we did not observe any mutant phenotype in vitro for the ECP1-deficient strain. Second, the genes encoding the extracellular proteins that are cloned so far are not clustered (R. Laugé, P. Vossen, and P. J. G. M. De Wit, *unpublished*). Third, we did not observe any qualitative change other than the absence of ECP1 in polyacrylamide gel electrophoresis profiles of extracellular proteins from plant material infected with the *Ecp1* mutant. We obtained *Ecp1* replacement with a frequency of ca. 1%, similar to frequencies reported for *Avr9* and *Ecp2* (Marmeisse et al. 1993, 1994). The same technique was successfully used to obtain a double replacement mutant. The same regulatory sequences used for the hygromycin B resistance gene in the *Ecp1* replacement cassette and for the

phleomycin resistance gene in the *Ecp2* replacement cassette (Marmeisse et al. 1994) might account for the lower replacement efficiency (ca. 0.25%) in obtaining the double *Ecp1/Ecp2* replacement mutant. Nevertheless, one double replacement mutant was recovered that showed the same stable characteristics as the single *Ecp*-replaced mutants. Thus, successive gene replacement is possible in the genome of *C. fulvum* and allows study of simultaneous knock-outs of several genes in the asexual fungal pathogen *C. fulvum*.

ECP1 and ECP2 are virulence factors of *C. fulvum*.

Disease development on 2-week-old seedlings axenically grown, inoculated, incubated in closed jars, and challenged with the ECP1-deficient strain indicated that the *Ecp1* gene was not essential for pathogenicity, as had already been reported for the *Ecp2* gene (Marmeisse et al. 1994). However, upon inoculation of 6-week-old plants in the glasshouse, we found a quantitative reduction in parasitic abilities of the ECP1- and ECP2-deficient strains. The ECP1-deficient strain exhibited normal growth in extracellular spaces of tomato leaves but showed decreased sporulation, whereas the ECP2-deficient strain grew less abundantly inside the leaf tissue and produced little emerging mycelium. Therefore, lack of ECP1 or ECP2 during infection results in abnormal pathogenesis on tomato without, however, abolishing pathogenicity. Thus, the two ECPs can be regarded as virulence factors of *C. fulvum*. It appears essential that pathogenicity assays with *C. fulvum* are carried out under conditions that resemble natural infections.

The double ECP1/ECP2-deficient strain is still pathogenic and exhibits a phenotype similar to that of the ECP2-deficient strain. This indicates that lack of one of the two proteins cannot be compensated for by the presence of the other one, as previously hypothesized (Marmeisse et al. 1994). Each ECP must fulfill a distinct function during the interaction with tomato. Consistent with our observation that lack of ECP2 leads to a more severe decrease of virulence for *C. fulvum* than lack of ECP1, the double-deficient strain resembles the ECP2-deficient strain. Subtle differences may exist between the ECP2-deficient and the double-deficient strains, but if so our pathogenicity assay was not sensitive enough to detect them.

Mixed inoculations of mutants and wild-type strains indicated that each ECP-deficient strain could only be partially complemented by ECPs produced by the wild-type strain. Mutation in ECPs could occur in nature but loss of either of the two proteins in natural populations might be counter selected for because of their lowered virulence and reproductive abilities.

Possible functions of ECP1 and ECP2 in pathogenesis.

C. fulvum does not form any specialized structures to colonize tomato leaves and does not cause detectable degradation of the plant tissue. Colonization by *C. fulvum* is restricted to the intercellular spaces, without visibly deleterious effects on the plant cells, indicating that this fungus does not require classical virulence and pathogenicity factors such as cell wall–degrading enzymes and toxins. The virulence functions provided by ECP1 and ECP2 remain to be determined. On the one hand, lack of ECP1 or ECP2 production affects *C. fulvum* in planta (low sporulation for the ECP1-deficient strain, poor colonization and little emerging mycelium for the ECP2-deficient strain). These effects might be due to their implication in metabolic functions such as nutrient uptake or substrate conversion. On the other hand, the ECP-deficient strains trigger stronger plant defense responses such as accumulation of extracellular PR proteins. The latter observations on the plant defense responses might point to a role in counteracting part of active plant defense responses. Possibly, lack of ECPs decreases fitness of the fungus that allows the plant to defend itself more efficiently. However, an alternative is that lack of ECPs does not allow the fungus to suppress the plant defense responses that hamper fungal development in the host when they reach a certain threshold. The latter hypothesis seems more likely, based on our observations that the ECP-deficient strains seem to fully regain pathogenic capacities, including sporulation on detached leaflets, and that they are not affected in growth and sporulation in vitro. Therefore, a role in attenuation of the plant defense responses for the ECPs during the interaction with the host is more plausible. The observation of unusually strong physiological responses such as collapse of guard cells is reminiscent of some characteristics of
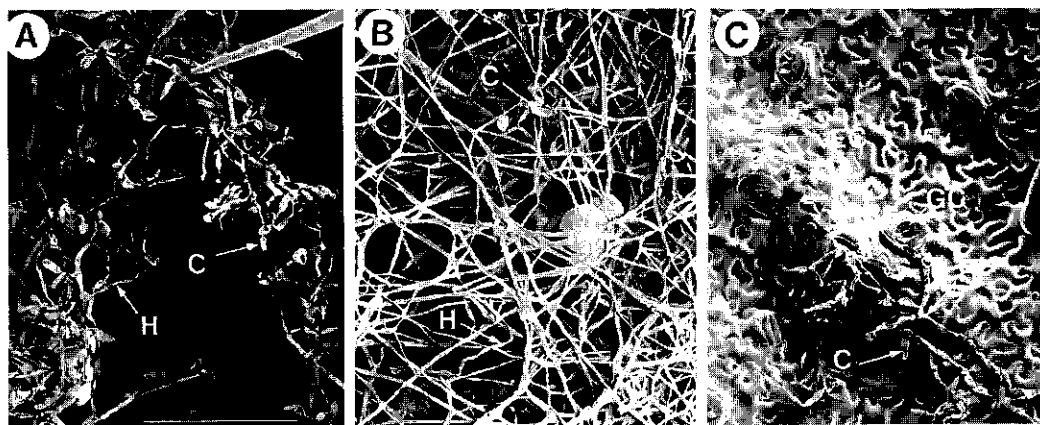

Fig. 5. Scanning-electron microscopic image of the lower side of tomato leaflets, 3 weeks after inoculation with (A) wild-type, (B) ECP1-deficient, and (C) ECP2-deficient strains. Stomatal guard cells (GC), epidermis cells (EC), fungal hyphae (H), and conidiophores (C) are indicated. Bar = 100 μm

the hypersensitive response. In immune reactions between avirulent strains of *C. fulvum* and resistant tomato genotypes, quick collapse of the host cells that come into contact with fungal hyphae is thought to be part of the successful arrest of fungal growth in early stages (Lazarovits and Higgins 1976). Similarly, rapid necrosis of stomatal guard cells has recently been proposed to be a crucial factor in resistance of Indian mustard cultivars toward *Leptosphaeria maculans* (Chen and Howlett 1996).

The sequence of the *Ecp*1 and *Ecp*2 genes did not provide any clues to putative functions (Van den Ackerveken et al.

1993). However, at the protein level, the spacing of the cysteine residues of ECP1 shows similarity to the cysteine spacing in the family of the tumor necrosis factor receptors (TNFRs) (Bazan 1993). Combined with our observations, an interesting parallel can be drawn with mammalian systems. Several mammalian viruses have been reported to produce extracellular suppressors of host defense responses (Gooding 1992). They act on cytokines, protein mediators of the immune system, a number of which are produced by host cells upon infection, including the tumor necrosis factors (TNFs). These extracellular suppressors share structural homologies with the host cell membrane–bound receptor that senses the cytokine signal. Due to this feature, they act as analogs of the host membrane–bound receptors and trap the cytokines before they reach their cellular target (Alcami and Smith, 1992; Spriggs et al. 1992). In this way, they interfere with cytokine function and disturb the establishment of host defense responses. Results of experiments carried out with viruses defi-
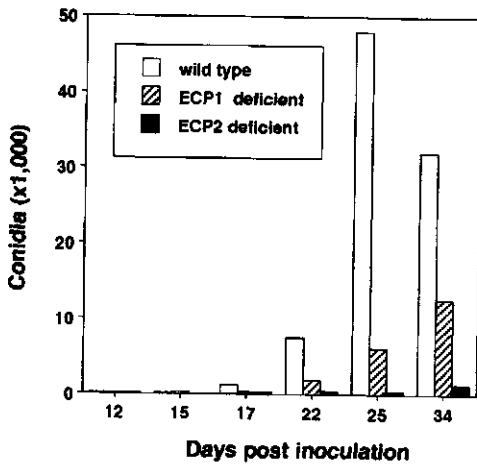

Fig. 6. Quantification of conidia isolated from six leaflets sampled from plants inoculated with wild-type, ECP1-deficient, and ECP2-deficient strains at several time points after inoculation.
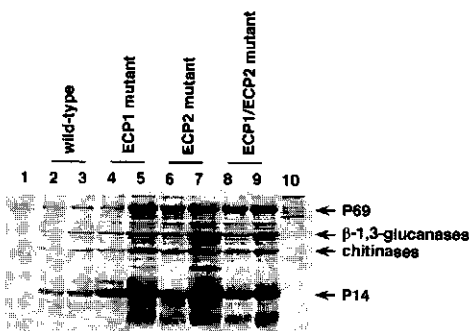

Fig. 7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of 100 µl of apoplastic washing fluid (AF) isolated from plant material 12 and 20 days post inoculation, respectively. Lanes 2 and 3, wild-type race 5; lanes 4 and 5, ECP1-deficient strain; lanes 6 and 7, ECP2-deficient strain; lanes 8 and 9, ECP1/ECP2-deficient strain. Lanes 1 and 10 contain AF isolated from noninoculated plants, obtained 12 and 20 days after initiation of the experiment, respectively. The most abundant pathogenesis-related proteins that accumulate in the apoplastic space during disease development are indicated on the right side (P69, β-1,3-glucanases, chitinases, and P14).
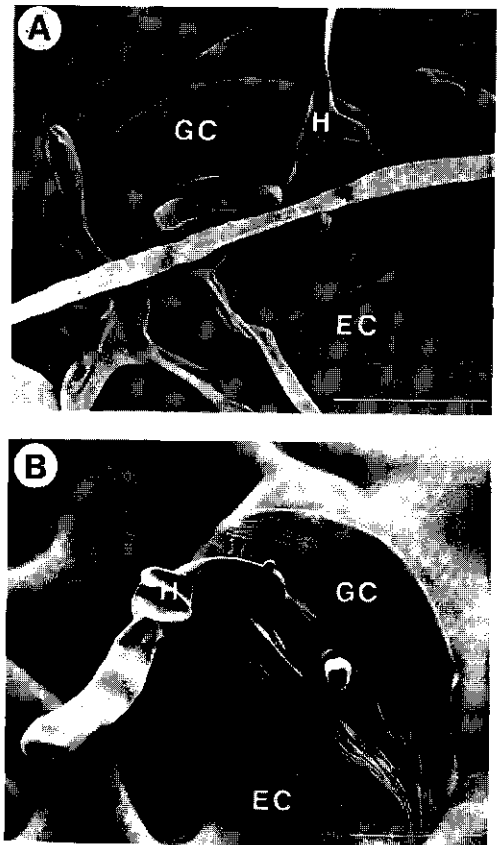

Fig. 8. Scanning-electron microscopy of stomatal guard cells at site of emergence of hyphae. Observations were made on plant material 3 weeks after inoculation with (A) wild-type and (B) ECP2-deficient strains. Stomatal guard cells (GC), epidermis cells (EC), and fungal hyphae (H) are indicated. Bar = 10 µm.

cient in these suppressor proteins show striking similarities to the results we obtained with tomato plants inoculated with the ECP-deficient strains of *C. fulvum*. Increased host defense responses were associated with lower pathogenic abilities of the deficient viral strain (Mossman et al. 1996). Notably, one of these suppressors, the protein T2 of the Shope Fibroma virus, shares the same type of structural homology with the TNFR family as ECP1, and was shown to competitively inhibit the binding of TNF to its cell surface receptor. Testing for the existence of a homologous situation in the interaction between *C. fulvum* and tomato becomes very attractive since the recent report on the cloning of a putative plant receptor that shares structural homology with the TNFR family (Becraft et al. 1996).

MATERIALS AND METHODS

Fungal strains and culture.

Procedures for handling fungal strains were those of De Wit and Flach (1979). Strains were routinely maintained on potato dextrose agar (Merck, Darmstadt, Germany). CDA (Czapek Dox agar, Oxoid, Basingstoke, UK) containing 100 μg of hygromycin B per ml (Sigma, Bornem, Belgium) was used for selection of the transformants.

Fungal transformation procedure.

Protoplasts of *C. fulvum* were prepared according to Harling et al. (1988) with the modifications of Van den Ackerveken et al. (1992). Mycelium of a 2-day-old B5 (Duchefa, Haarlem, The Netherlands) liquid culture of the recipient race 5 of *C. fulvum* was digested in MM (20 mM 2-[*N*-morpholino]ethanesulfonic acid, pH 5.8, 1 M MgSO$_4$, containing 5 mg of Novozym 234 [Novo Biolabs, Cambridge, UK] per ml). Chemical transformation was achieved with polyethylene glycol 6000 (Merck) according to Oliver et al. (1987). Protoplasts (1 to 5 × 10^6) were mixed with 10 μg of the plasmid pCF148, carrying the *Ecp*1 replacement cassette, in MTC (1 M MgSO$_4$, 10 mM CaCl$_2$, 10 mM Tris-HCl, pH 7.5). Fifteen minutes post incubation at room temperature, 1 vol of PTC (20% polyethylene glycol 6000, 10 mM CaCl$_2$, 10 mM Tris-HCl, pH 7.5) was added. After incubation for 15 min at room temperature, 5 vol of CM (Czapek Dox salts [Oxoid] 33.4 g/liter, potato dextrose broth [Difco, Detroit, MI] 12 g/liter, yeast extract [Oxoid] 1 g/liter, mycological peptone [Oxoid] 1 g/liter, casein hydrolysate [Gibco BRL, Breda, The Netherlands] 1 g/liter, sucrose [Merck] 273.84 g/liter) in top agar were added. The mixture was then plated on CM selection plates containing 100 μg of hygromycin B per ml. Hygromycin B resistant transformants were obtained after 3 to 4 weeks of culture.

Plants and inoculations.

Two-week-old tomato seedlings were grown on Hoagland Agar in vitro. Six-week-old tomato plants were grown in the glasshouse in 1.5-liter pots. Both seedlings and plants were inoculated with an aqueous conidial suspension of 5 × 10^6 per ml. The same inoculum pressure was applied for the co-inoculation experiments by mixing 1 vol of a wild-type conidial suspension of 5 × 10^6 per ml with 1 vol of a mutant conidial suspension of 5 × 10^6 per ml prior to inoculation. Seedlings were briefly dipped into the solution, whereas plants were inoculated as described by De Wit (1977). Symptoms on

seedlings were recorded 2 weeks after inoculation and on adult plants from 2 weeks after inoculation and onward. The inoculation experiments of seedlings and adult plants were conducted five times. Conidial inocula were checked for germination percentage on solid medium.

Cloning procedures.

All plasmid DNA manipulations were carried out essentially as described by Sambrook et al. (1989). Restriction and modifying enzymes (Gibco BRL) were used for digestions according to the manufacturer's recommendations. DNA fragments were separated on Ultrapure agarose (Gibco BRL) gels and purified with the GlassMAX DNA Isolation System (Gibco BRL).

PCR on conidia.

Putative *Ecp*1 replacement mutants of the Δ*Ecp*2-D39 strain were prescreened by PCR, which was performed on conidia. We followed the procedure described for *Magnaporthe grisea* by Xu and Hamer (1995). The two 23-base oligonucleotides, ECP1.1 (5'-TGA GTT GCG GAA CTT CGC GGA GC-3') and ECP1.2 (5'-GTG GCA TTC AGC CTC CGC GAT GC-3'), allowed amplification of a unique 696-bp fragment for transformants that still carry a wild-type *Ecp*1 locus.

DNA isolation and analyses.

Genomic DNA of *C. fulvum* was isolated according to Van Kan et al. (1991). Freeze-dried mycelium was ground into a fine powder and homogenized in extraction buffer (0.5 M NaCl, 1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 10 mM Tris-HCl, pH 7.5). The mixture was extracted three times with phenol/chloroform/isoamyl alcohol (24/24/2; vol/vol/vol) and once with chloroform/isoamyl alcohol (24/1; vol/vol). The nucleic acids present in the aqueous phase were precipitated with 1 vol of isopropanol and submitted to RNAseA digestion for 30 min. Purified DNA was obtained after repeated chloroform/isoamyl alcohol extraction, and digested with restriction enzymes. The digested DNA was separated on 0.8% agarose (Gibco BRL) TAE (0.04 M Tris-acetate, 1 mM EDTA pH 8.0) gels and transferred onto Hybond N$^+$ nylon membranes (Amersham, Buckinghamshire, UK) with a vacuum blotter (Millipore, Etten-Leur, The Netherlands). Hybridization procedures were performed overnight at 65°C in 5× Denhardt's reagent, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 0.5% (wt/vol) SDS. Washing steps were done at 65°C, once for 30 min in 2× SSC, 0.1% (wt/vol) SDS, and twice for 1 h in 0.2× SSC, 0.1% (wt/vol) SDS. Probes were radiolabeled with the Random Primers Labeling System (Gibco BRL) and 30 μCi α^{32}P-dATP (Amersham).

Light and scanning-electron microscopy.

All microscopy studies were performed on 1-cm-diameter leaf disks that were sampled randomly from the infected area at different time points after inoculation. For cytological examination, samples were cleared in Farmer's fluid (acetic acid/ethanol/chloroform [1/6/3; vol/vol/vol]), stained in lactophenol (lactic acid/glycerol/water [1/1/1; vol/vol/vol]) containing 30% (wt/vol) phenol and 0.5% (wt/vol) cotton blue. After destaining in chloral hydrate (2.5 g/ml), samples were observed by light microscopy with an Axioskop microscope (Zeiss, Oberkochen, Germany). For cryo-scanning-electron

microscopy, samples were frozen in liquid nitrogen, sputter coated with palladium gold, and observed in a JEOL JSM 5200 (Jeol, Tokyo).

Quantification of conidia production and germination of conidia.

To quantify production of conidia, six leaflets per plant were randomly picked from a uniformly diseased area at various time points after inoculation. They were submerged in water in a 50-ml screw cap tube (Greiner, Alphen aan de Rijn, The Netherlands) and placed on a rolling bench (Greiner) for 15 min to allow the majority of the conidia to become suspended. The leaflets were discarded, and the suspended conidia were pelleted at $3,000 \times g$ and counted in a hemacytometer cell (W. Schreck, Hofheim, Germany). After the mixed inoculations, conidia were quantified in the same way. The identity of the conidia was determined by differential plating on CDA for the total amount of conidia, CDA containing 100 μg of hygromycin B per ml for the *Ecp*1-replaced strain, and CDA containing 20 μg of phleomycin (Cayla, Toulouse, France) per ml for the *Ecp*2-replaced strain. As germination efficiencies of hygromycin B– and phleomycin-resistant conidia differed on media either containing the corresponding antibiotic or not, corrections were made.

Isolation and analysis of apoplastic fluids.

For the different time-course experiments, apoplastic fluids were isolated at various time points after inoculation. For each plant, six infected leaflets were randomly chosen and AF was obtained by in vacuo water infiltration followed by centrifugation at $3,000 \times g$ according to De Wit and Spikman (1982). Protein profiles were analyzed on 15% (wt/vol) polyacrylamide slab gels under native conditions or denaturing conditions in the presence of SDS. Western blot analyses were carried out after electro transfer of the separated proteins on nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany). Polyclonal antibodies against ECP1 (Joosten and De Wit, 1988), against ECP2 (Wubben et al. 1994), and against ECP3 (M. H. A. J. Joosten, R. Laugé, and P. J. G. M. De Wit, *unpublished*) were raised in rabbits. Polyclonal antibodies against AVR4 (Joosten et al. 1997) and against AVR9 (P. J. G. M. De Wit, P. Vossen, and M. H. A. J. Joosten, *unpublished*) were raised in mice. GARAP and GAMAP antibodies were obtained from Bio-Rad (Veenendaal, The Netherlands). ELISAs were performed on Nunc-Immuno Plate MaxiSorp (Nunc, Roskilde, Denmark). Optical density was read at 405 nm in an EL312 microplate reader (Bio-Tek, Winooski, VT).

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Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen

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ABSTRACT The interaction between tomato and its fungal pathogen *Cladosporium fulvum* complies with the gene-for-gene system, in which specific recognition of fungal proteins by plant genotypes with matching resistance genes results in host resistance. Two proteins, ECP1 and ECP2, secreted by *C. fulvum* during infection, are required for full virulence of the fungus on tomato. We chose the most important virulence factor, ECP2, for a targeted search for hypersensitive response (HR)-based resistance among a collection of tomato genotypes. By screening with recombinant potato virus X that expresses the *Ecp2* gene, we identified four lines that respond with HR toward ECP2. The capacity to recognize ECP2 and induce HR is sufficient to confer resistance in tomato against *C. fulvum* producing ECP2. Resistance is based on a single dominant gene, which we have designated *Cf-ECP2*, for resistance to *C. fulvum* through recognition of ECP2. Accordingly, an *Ecp2*-minus strain created by gene replacement is pathogenic on *Cf-ECP2* plants. However, due to lack of ECP2 the mutant strain is only weakly virulent. All strains of a worldwide collection of *C. fulvum* strains that were tested were found to produce a HR-inducing ECP2 protein. Because the *Cf-ECP2* gene operates through recognition of an important virulence factor, we expect it will confer durable resistance against *C. fulvum*. A similar targeted approach should allow the discovery of new valuable resistance genes in other pathosystems.

Plant surfaces and intercellular spaces are subjected continuously to potential pathogens. However, individual host plants that exhibit genetic resistance to a particular pathogen often occur in nature. Plant breeders have exploited natural resistance genes extensively via introgression from wild species into high-yielding agronomic cultivars. Nevertheless, this strategy has become less successful as resistance sources are limited and most plant pathogens show remarkable genetic variation leading to the appearance of strains that overcome introgressed genetic resistances (1). Most of the recognition mechanisms in plants remain unrevealed, and the practice of resistance breeding is rather empirical (2). In animal systems, viral vectors are used routinely to deliver antigens to raise antibodies against important pathogens (3–5). Often these antigens are derived from virulence factors of those pathogens. Following a similar approach, plant viruses can be used to deliver virulence factors of plant pathogens to find plants responding with a hypersensitive response (HR). Such plants would likely exhibit a durable type of HR-resistance toward the pathogen from which the virulence factor is originating.

The *C. fulvum*–tomato (*Lycopersicon esculentum*) interaction is a well established model system that complies with the gene-for-gene relationship (6). It is also one of the few systems in which there is ample experimental evidence for the involvement of proteinaceous elicitors (avirulence factors) from the pathogen in the induction of active HR-related resistance (7). Many HR-resistant traits have been described in accessions of wild *Lycopersicon* species and several dominant resistance genes have been introgressed into modern tomato cultivars. HR-mediated resistance in tomato against *C. fulvum* manifests itself as death of the first cells that come into contact with the penetrating fungus. Further growth of the pathogen is prevented, and the interaction is incompatible. In contrast to resistant plants, susceptible plants do not exhibit HR during interaction with the fungus. Colonization by fungal hyphae occurs through the whole leaf tissue and the interaction is compatible (8). As the development of *C. fulvum* is restricted to the apoplast of the leaf mesophyll, the elicitors that are secreted by the fungus can be isolated from apoplastic washing fluids (AFs) (9). In this way, two proteinaceous avirulence factors, AVR9 and AVR4, responsible for fungal recognition by host genotypes carrying the matching resistance genes *Cf-9* and *Cf-4*, respectively, have been characterized (10, 11). Two additional fungal extracellular proteins, ECP1 and ECP2, occur abundantly in AF of plants infected by *C. fulvum* (12,13). Analysis of mutants of the fungus, in which either the *Ecp1* gene or the *Ecp2* gene had been deleted, showed that both ECP proteins are virulence factors for *C. fulvum*. ECP2 is the most important of the two because *Ecp2*-lacking strains are only weakly pathogenic, exemplified by poor leaf colonization and conidiation (14). By using the potato virus X (PVX) expression system (15), we have identified tomato lines that display HR upon exposure to ECP2. The gene, designated *Cf-ECP2*, which is responsible for ECP2 recognition confers resistance against *C. fulvum* on tomato. Because of the crucial role of ECP2 in virulence of the fungus, *Cf-ECP2* may prove to be of increased durability and would therefore be valuable in breeding programs aimed at sustainable agriculture.

MATERIALS AND METHODS

Construction of Recombinant PVX::*Ecp2* and PVX Inoculation Procedure. The chimeric construct for *Ecp2* expression and extracellular targeting of the ECP2 protein was obtained by PCR-mediated cloning. The two pairs of oligonucleotides PR1ECP2F (5′-CTTGCCGTGCCCGGAACGCTGGCAA-CTCGCCC-3′) and ECP2CLA (5′-CGGAAGCTTATCGA-TCTAGTCATCGTTGGACGGGTTG-3′); and OX10 (5′-CAATCACAGTGTTGGCTTGC-3′) and PR1ECP2R (5′-G-TTGCCAGCGTTCCGGGCACGGCAAGAGTGGGATA-TTAC-3′) were used for PCR with the *Ecp2* cDNA and the PVX::*Avr4* construct as templates, respectively (16). After

PCR overlap extension and cloning in the PVX vector (15), the final PVX construct carried a chimeric transgene consisting of the sequence coding for the PR-1a plant signal peptide fused to the *Ecp2* cDNA encoding the mature protein. This fusion was placed under the control of the PVX coat protein promoter, enabling expression as a subgenomic messenger during virus spread throughout the whole plant. Full-length infectious transcripts were generated *in vitro* by using the T7 mMES-SAGE mMACHINE kit (Ambion, Austin, TX) according to the recommendations of the supplier. The transcripts were inoculated by rubbing onto the leaves of *Nicotiana clevelandii* plants in presence of carborundum. Ten days after inoculation, infected leaves of *N. clevelandii* were collected and ground in 50 mM potassium phosphate buffer, pH 7.7, to prepare sap, which contains recombinant virus particles. The sap was used subsequently for inoculation of tomato lines in a similar way as described for *N. clevelandii*.

C. *fulvum* Inoculation Procedure. Suspensions of conidia of *C. fulvum* were prepared as described (17). In brief, fungal strains were grown on potato dextrose agar plates and conidia were suspended in water by rubbing the sporulating colony. The suspension that was obtained was sprayed onto the lower side of the tomato leaves. Disease symptoms were scored 2–3 weeks after inoculation.

Isolation, Purification, and Immunodetection of ECP2. Isolation of AFs from susceptible tomato plants inoculated with various *C. fulvum* strains was performed as described (9). Two weeks after inoculation, infected leaves were collected and water infiltrated in a vacuum chamber, and AF-containing soluble apoplastic compounds was obtained after centrifugation at 3,000 × g for 10 min. Purified ECP2 was obtained from AF of a compatible *C. fulvum*–tomato interaction by gel filtration on a Sephadex G-50 column (Pharmacia) followed by chromatography on a Resource Q column (Pharmacia) as described by Wubben *et al.* (13). ECP2-containing fractions were identified with immunodetection by using polyclonal antibodies raised against ECP2 (13). For immunodetection of ECP2, AF containing the extracellular proteins produced *in planta* by the various strains that were tested, was separated on 15%-polyacrylamide gels containing SDS. The separated proteins were subsequently electro-transferred to Immobilon-P

membrane (Millipore), and the blots were incubated with ECP2 polyclonal antibodies.

Crossings. To obtain three F_1 generations, lines 1 and 4 and their common ancestor were crossed to the nonresponsive Moneymaker cultivar, using the latter cultivar as the female parent. These F_1 generations were selfed to obtain the F_2 generations used in this study.

RESULTS

Identification of Tomato Genotypes that Display HR upon Exposure to ECP2. We have screened 21 lines that originated from early breeding programs for resistance against *C. fulvum*. They had been selected for resistance against the tester strain, race 2.3.4, which overcame all resistance genes, *Cf-2*, *Cf-3*, and *Cf-4*, that had been introgressed at that time (18, 19). The lines that should carry resistance genes different from these three genes were tested for the ability to respond with HR after exposure to ECP2. Screening was carried out by using PVX (15) for systemic production of the ECP2 protein, targeted to the apoplast of virus-infected plants (16), and results were verified with purified native ECP2 protein. PVX::*Ecp2* recombinant virus was obtained by cloning the *Ecp2* cDNA encoding the mature ECP2 protein, downstream of the sequence encoding the PR-1a plant signal peptide, under the control of the PVX coat protein promoter (Fig. 1*A*). Native ECP2 protein was obtained by sequential chromatographic separation of soluble proteins present in AF from a compatible *C. fulvum*–tomato interaction (Fig. 2*A*). Four of 21 tomato lines that were tested responded with HR after inoculation with PVX::*Ecp2*, as well as after injection with native ECP2. PVX::*Ecp2* inoculation triggered the development of systemic chlorotic and necrotic lesions in leaves of the four lines (Fig. 1*B*). Upon injection of ECP2, the four lines developed chlorosis followed by necrosis in the center of the injected area within 3 days (Fig. 2*B*). Inoculation of these four lines with wild-type PVX caused normal mosaic symptoms. The other 17 lines and the control cultivar Moneymaker (lacking any known *C. fulvum* resistance gene) exhibited normal mosaic symptoms upon inoculation with either PVX::*Ecp2* or wild-type PVX. Systemic HR never was observed in these plants. These results indicate that ECP2
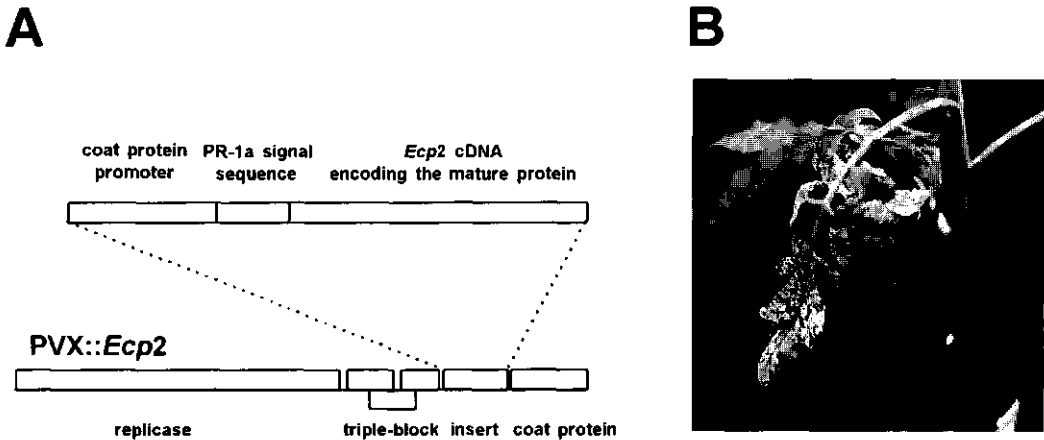
A

coat protein promoter PR-1a signal sequence *Ecp2* cDNA encoding the mature protein

PVX::*Ecp2*

replicase triple-block insert coat protein

B

FIG. 1. Construction of the PVX::*Ecp2* derivative and identification of lines responding with HR upon exposure to the ECP2 protein. (*A*) Schematic map of the PVX::*Ecp2* derivative. (*B*) Specific HR upon ECP2 presentation. Four-week-old plants of the collection of tomato lines and the control cultivar Moneymaker (lacking any known *C. fulvum* resistance gene) were inoculated with sap containing PVX::*Ecp2* (16) and symptoms were recorded 10–14 days after inoculation. Control plants of each line were inoculated with sap containing wild-type PVX. All plants gave normal systemic mosaic symptoms upon inoculation with wild-type PVX. Four lines gave systemic chlorosis and necrosis upon inoculation with PVX::*Ecp2*. The symptoms on one of these plants, line 1, are shown. Normal mosaic symptoms developed after inoculation with PVX::*Ecp2* on all other plants.
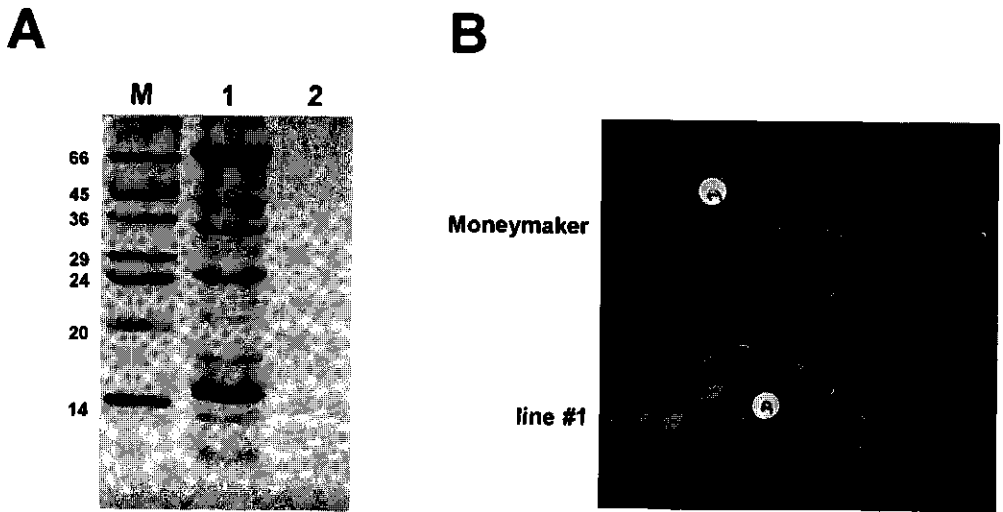
FIG. 2. HR induced after injection of purified ECP2 in a leaflet of line 1. (A) Purification of the ECP2 protein. Lane M contains molecular weight markers, lane 1 contains 50 μl of AF, and lane 2 contains the purified ECP2 protein. (B) HR induced after injection of ECP2 in the apoplast of a leaflet of a line 1 plant (Lower) and absence of HR after injection of ECP2 in the apoplast of a leaflet of the Moneymaker cultivar (Upper). Only the four lines identified by inoculation with PVX::Ecp2 responded by chlorosis, followed by necrosis in the injected area three days after injection of ECP2; all other lines showed no detectable response after ECP2 injection.

is both necessary and sufficient to induce HR on the four responding lines.

ECP2-Mediated HR Is Determined by a Single Dominant Gene. The four independent lines that recognize ECP2 have been reported to originate from the same *L. pimpinellifolium* ancestor and are likely to be four independent introgressions containing the same resistance factor (18, 19). We produced three F_1 progenies by crossing line 1, line 4, and their ancestor to the cultivar Moneymaker. F_2 progenies were generated to study the heritability of HR upon exposure to ECP2. We inoculated PVX::*Ecp2* onto F_1 plants and three F_2 populations containing 93, 105, and 85 individuals, respectively, and determined the segregation ratio for HR. All F_1 individuals showed HR, and the three F_2 populations exhibited a 3:1 segregation for presence to absence of HR after inoculation with PVX::*Ecp2* (Table 1). This demonstrates that one dominant gene determines the capacity to develop HR upon exposure to ECP2 in the ancestor and lines 1 and 4.

ECP2 Recognition Confers Resistance Against *C. fulvum*. We used isogenic strains of *C. fulvum*, either with or without the *Ecp2* gene, to prove that resistance of these responding lines is solely caused by the fact that the fungus produces ECP2 during infection. We used line 1 as a representative of the four lines. The set of fungal strains tested comprised a wild-type, ECP2-producing strain (race 5), the corresponding isogenic *Ecp2*-lacking mutant in which the *Ecp2* gene had been replaced

by an antibiotic resistance cassette, and an ECP2-overproducing strain obtained after retransformation of the *Ecp2*-lacking mutant with multiple copies of the *Ecp2* gene (20). Whereas susceptible Moneymaker plants showed disease symptoms when inoculated with either of the three strains, only the *Ecp2*-lacking mutant caused disease on line 1 (Fig. 3). Plants of line 1 showed full resistance without any visible disease symptoms after inoculation with the wild-type ECP2-producing strain or the ECP2-overproducing near-isogenic strain. Microscopic examination of these resistant plants confirmed that fungal growth of both the wild-type and the ECP2-overproducing strains is arrested early after penetration of the tomato leaf. The *Ecp2*-lacking strain colonized to the same low extent the mesophyll of either plants of line 1 or Moneymaker plants (data not shown). The latter observation confirms the role of ECP2 as a virulence factor of *C. fulvum*

Table 1. Inheritance of HR induced by ECP2

	F_1		F_2		
	HR	Mosaic	HR	Mosaic	χ^2 Value
Line 1 × Moneymaker	4	0	77	16	1.13 ($P > 0.25$)
Line 4 × Moneymaker	5	0	82	23	0.27 ($P > 0.5$)
Ancestor × Moneymaker	4	0	67	18	0.25 ($P > 0.5$)

Segregation of HR induction by ECP2 in F_1 and F_2 generations originating from a cross between the HR-displaying line 1, line 4, and their common ancestor with the non-HR-displaying cultivar Moneymaker. Plants were inoculated with PVX::*Ecp2* and scored for systemic HR 2 weeks after inoculation.

ECP2-producing wild-type   ECP2-deficient mutant   ECP2-overproducing mutant

FIG. 3. Resistance of *Cf-ECP2* lines is solely dependent on the production of the ECP2 protein by *C. fulvum* during infection. Plants of line 1 were inoculated with the wild-type ECP2-producing strain (race 5) (Left), the isogenic *Ecp2*-lacking strain (Center), and the ECP2-overproducing strain (Right). Note that only the plants inoculated with the *Ecp2*-lacking strain showed symptoms of chlorosis on the upper side of the leaf 20 days after inoculation.

on tomato as described before (14). Thus, resistance toward *C. fulvum* of lines 1 to 4 is solely dependent on recognition of the ECP2 protein, as was expected from the results obtained with the PVX::*Ecp2* experiments. We designate this resistance gene *Cf-ECP2* because this single dominant gene confers resistance toward *C. fulvum* through recognition of the ECP2 protein.

Strains of *C. fulvum* that Have Been Collected Worldwide Produce a HR-Inducing ECP2 Protein During Infection. To test the effectiveness of the *Cf-ECP2* gene, we analyzed AFs that were obtained from susceptible plants infected by a worldwide collection of 25 strains of *C. fulvum*, for the presence of the ECP2 protein and its HR-inducing activity. Western blot analysis with polyclonal antibodies raised against ECP2 confirmed the production of ECP2 by all strains (Fig. 4). Differences in accumulation levels of ECP2 are in agreement with accumulation levels of other *in planta*-secreted proteins of the various *C. fulvum* strains and reflect their relative aggressiveness. In addition, leaf injection of line 1 with AFs from all 25 strains resulted in the induction of HR, indicating that all strains produce an elicitor-active ECP2 protein (Fig. 4).

DISCUSSION

Resistance breeding programs are traditionally based on the identification of resistance to a pathogen in wild relatives, followed by several generations of backcrossing to introduce the resistance into elite breeding lines. Initially, inoculations with local and worldwide strains of the pathogen give insight into the effectiveness of the resistance (2). HR-based resistance can be induced by a variety of elicitors originating from the pathogen, of which the importance in pathogenicity or virulence is not known beforehand. If the elicitors are not important pathogenicity or virulence factors, it is likely that the pathogen will easily overcome the resistance either by mutating or losing the encoding gene. Here, we exploited biochemical and molecular data concerning a factor that is important for full virulence of the pathogen and followed a targeted search for plants showing a HR response to this factor.

Frequently, new races of *C. fulvum* appear that overcome known resistance genes such as *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9*. Molecular analysis of strains of *C. fulvum* that circumvent the *Cf-9* resistance gene revealed that complete deletion of the
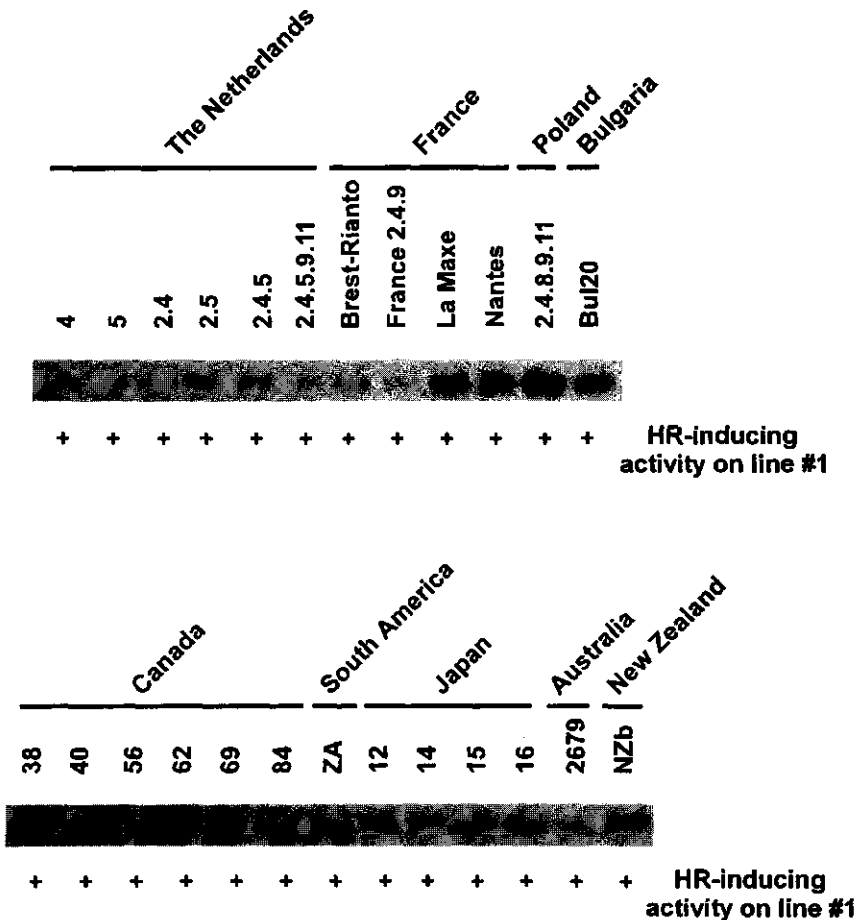

FIG. 4. Immunodetection of ECP2 in AFs isolated from cultivar Moneymaker inoculated with 25 strains of *C. fulvum* obtained from a worldwide collection. A volume of AF containing 4 µg of protein was separated on denaturing polyacrylamide gel, blotted, and incubated with polyclonal antibodies raised against ECP2. ECP2 is detected in each lane. HR-inducing activity of each AF sample was assayed by injecting 5–10 µl into a leaflet of line 1. HR activity visible 3 days after injection is indicated by "+."

Avr9 gene had occurred in the fungal genome, with the consequence that recognition is avoided as the AVR9 elicitor is no longer produced (21). *C. fulvum* strains that circumvent the *Cf-4* resistance gene exhibit a single point mutation in the *Avr4* gene, which causes instability of the encoded product, because no proteins homologous to AVR4 are detected in AF of plants inoculated with these strains (11, 16). So far, no intrinsic biological function has been assigned to the *Avr9* and *Avr4* genes of *C. fulvum*. It seems that these genes can be lost without a detectable fitness penalty, so they appear not to encode important factors of pathogenicity or virulence for *C. fulvum*.

The *Cf-ECP2* resistance gene matches an important virulence factor of *C. fulvum* (14), which is present in all strains of *C. fulvum* that have been tested up till now. Overexpression of *Ecp2* in plants lacking *Cf-ECP2* by using the PVX expression system does not give a phenotype, which might be explained by the fact that ECP2 rather suppresses activation of plant defense responses than being a toxic virulence factor. So far, four bacterial and one fungal avirulence gene (*avrA* and *avrE* from *Pseudomonas syringae* pv. *tomato*, *avrRPM1* from *Pseudomonas syringae* pv. *maculicola*, *avrBs2* from *Xanthomonas campestris* pv. *vesicatoria*, and *nip1* from *Rhynchosporium secalis*; refs. 22–25) have been reported to possess a function in virulence. However, these genes were not discovered and isolated based on a targeted search as described here. The matching resistance genes in the host plant are expected to confer durable protection against these pathogens. We do not know whether there are separate domains present in the mature ECP2 protein of 142 amino acids, which are important for its virulence function and for the HR-inducing activity. If there are no separate domains in the ECP2 protein, or if the two separate domains overlap, the fungus cannot circumvent the *Cf-ECP2* resistance by mutations in the *Ecp2* gene, without a serious decrease in virulence. Altogether, we expect the *Cf-ECP2* resistance gene to be efficient and durable in protecting tomato crops against *C. fulvum*.

Using a viral expression system, such as the one based on PVX, has great potential. It ensures that the plant response is solely due to the protein of which the encoding cDNA has been inserted into the PVX vector. PVX screening can easily be performed with any protein. As in immunization assays used for mammals, the PVX vector presents the protein throughout the infected plant, increasing the amount of responding plant tissue from one single injection site to the whole plant, in which the virus systematically spreads (26). If the heterologous protein expressed by PVX triggers a quick HR response in the host, spread of the virus might be restricted to the inoculated area. In this case, compared with systemic mosaic symptoms caused by wild-type PVX, absence of mosaic symptoms combined with localized necrosis on the inoculated leaves may indicate recognition of the expressed protein. This method allows screening of large populations of wild plants or recombinants for rare individuals that exhibit specific recognition of the protein to which they have been exposed. We are currently testing large collections of wild *Lycopersicon* species for HR upon exposure to several additional potential virulence factors of *C. fulvum*. With the availability of several plant viral expression vectors for dicots and monocots, such as PVX, tobacco mosaic virus, cauliflower mosaic virus, tomato golden mosaic virus, cassava latent virus, brome mosaic virus, and barley stripe mosaic virus (15, 27–33), plants other than *Solanaceae* can be screened for HR-mediated resistance toward these viral vectors expressing pathogenicity or virulence factors produced by economically important plant pathogens.

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Chapter 5

Specific cell death-associated recognition

of secreted proteins from a fungal

pathogen occurs in host and

non-host plants

in preparation

SPECIFIC CELL DEATH-ASSOCIATED RECOGNITION OF SECRETED PROTEINS FROM A FUNGAL PATHOGEN OCCURS IN HOST AND NON-HOST PLANTS

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Different from animals, little is known about the defense mechanisms operating in plants when they are challenged by pathogens. Many plant-pathogen interactions comply with the gene-for-gene concept, for which host resistance is generally based on cell death-associated recognition of molecules from the intruding pathogen. *Cladosporium fulvum* is a fungal pathogen of tomato, that grows exclusively in the extracellular spaces of the leaves. It has been demonstrated that resistance against this fungal pathogen operates through specific recognition of secreted fungal proteins by plant cells, followed by a hypersensitive response (HR) which involves quick local death of host cells at the site of penetration. Here, we present evidence that, within the genus of tomato, individuals exist that specifically respond with HR to each of four newly identified proteins secreted by the fungus. We demonstrate that this phenomenon is also observed in tobacco, a non-host plant of *C. fulvum*. Moreover, this HR-associated recognition confers resistance against a viral pathogen of tobacco expressing the gene encoding one of the proteins. From our results we conclude that plants display a versatile HR-associated recognition system for foreign proteins, which provides the molecular basis for the gene-for-gene relationships frequently observed in natural pathosystems.

INTRODUCTION

Since the end of last century, genetic resistance of plants against their pathogens has been demonstrated. In various plant-pathogen interactions, inoculation of the pathogen onto wild relatives of the host plant often reveals individuals that can resist the pathogen. Most of these resistances were transferred to cultivated plants by crossing, which resulted in efficient protection against the pathogen. It also enabled to study the genetic basis of resistance. Many of these resistances were proven to inherit as monogenic dominant characters and were named "resistance genes" (*R* genes) (Biffen, 1905, 1907, 1912). Subsequently, many plant species of different families were demonstrated to possess *R* genes that are active against various types of pathogens, including viruses, bacteria, fungi and nematodes. Whilst rarely visible by the naked eye, microscopical examination of resistant plant tissues revealed death of the host cells around the site of penetration of the pathogen, a phenomenon named the hypersensitive response (HR). HR is believed to cause arrest of further growth of the pathogen and to be triggered through specific recognition of components of the invader by the host (Lamb, 1994).

In various cases it has been shown that one or more of the proteinaceous molecules produced by the pathogen upon infection is perceived as an foreign molecule by the resistant plant (De Wit, 1992), and that this recognition of an "avirulence factor" is conferred by the

presence in the host of a specific *R* gene (De Wit, 1992, 1997). Therefore, in theory, there could be as many resistance genes to a pathogen as the number of molecules it produces while attacking the plant. For a given plant-pathogen interaction, the *R* genes introduced in crop plants probably represent only a subset of the *R* genes existing in populations of wild relatives, especially as breeders have mostly introduced R genes with clear and strong resistance phenotypes.

The interaction between tomato (*Lycopersicon esculentum*, Miller) and its fungal leaf pathogen *Cladosporium fulvum* (Cooke) complies with the gene-for-gene system described by Flor (1946, 1955; Day, 1974). Several *Cf* (for resistance against *C. fulvum*) resistance genes, such as *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*, were detected at random in wild *Lycopersicon* species and were successfully introgressed in tomato. The confinement of the fungus to the intercellular space of the tomato leaves during its whole pathogenic cycle let us hypothesize that *Cf* genes would operate through perception of fungal molecules that are secreted by the fungus during colonization of the intercellular space (De Wit, 1992). Indeed, the analysis of the molecules present in apoplastic washing fluids of infected leaves led to the identification of two proteinaceous elicitors, AVR4 and AVR9, that specifically induce an HR when injected intercellularly in leaves of *Cf-4* and *Cf-9* plants, respectively. Molecular analysis of the genes encoding the two elicitors confirmed that they are the avirulence genes matching the two resistance genes (Van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). So far, it is not clear how many *R* genes effective against *C. fulvum* exist in wild relatives of tomato. However, identification of homologues of the *Cf-9* resistance gene, conferring resistance towards the fungus (Parniske *et al.*, 1997; Laugé *et al.*, 1998a; Takken *et al.*, 1998), and the success of a targeted search for the *Cf-ECP2* resistance gene operating through HR-associated recognition of ECP2, a factor which is essential for full virulence of the fungus (Laugé *et al.*, 1998b), suggests that *R* genes against *C. fulvum* occur frequently. As for this interaction, resistance operates through HR-associated recognition of extracellular fungal proteins, we hypothesized that any extracellular protein produced by the fungus during colonization, potentially functions as an epitopic determinant capable of inducing HR when recognized by particular plant genotypes.

Here we describe the isolation of ECP1 (Joosten and De Wit, 1988), ECP2 (Wubben *et al.*, 1994) and three additional extracellular proteins (ECPs) secreted by *C. fulvum* during colonization. Either by injection of the purified proteins or by screening with recombinant potato virus X (PVX) (Chapman *et al.*, 1992), expressing an ECP-encoding gene, on breeding lines and wild *Lycopersicon* species, we have identified, for each ECP, individuals that display HR upon exposure to the protein. The PVX expression system also allowed us to screen solanaceous plants that are not a host for *C. fulvum* for responsiveness to ECPs. Our results suggest that the gene-for-gene concept results from the widely occurring inherent capacity among plants to respond with HR to non-self molecules.

RESULTS

Isolation of five extracellular proteins (ECPs) of *C. fulvum*

Proteins present in apoplastic washing fluids (AFs) of compatible and incompatible *C. fulvum*-tomato interactions were separated by gel filtration, followed by anion exchange and reverse phase chromatography. The chromatographic profiles of AFs from a compatible interaction were compared with those obtained from an incompatible interaction, involving a plant resistant to the strain of *C. fulvum* that was used for inoculation. In the incompatible interaction, hardly any fungal growth occurs and as a consequence no secreted fungal proteins are observed. Five secreted fungal proteins were purified from AF originating from a compatible interaction (Figure 1). Two of these five proteins correspond to the extracellular proteins ECP1 (Joosten and De Wit, 1988) and ECP2 (Wubben *et al.*, 1994), from which the encoding genes have been cloned before (Van den Ackerveken *et al.*, 1993), whereas three additional extracellular proteins, named ECP3, ECP4 and ECP5 were isolated. The apparent molecular masses of ECP3, ECP4 and ECP5 were estimated to be 19kDa, 12kDa and 7kDa, respectively. Partial amino acid sequence information did not show homology to sequences reported in databases. Furthermore, these proteins do not share homology with each other, or with ECP1, ECP2, AVR4 or AVR9.
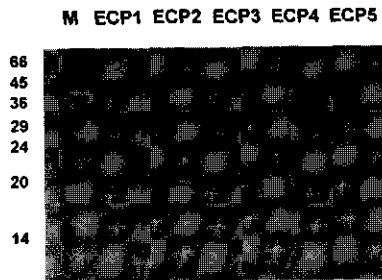

Figure 1. The five purified ECP proteins used in this study.
The proteins were separated by electrophoresis under denaturing conditions on sodium dodecyl sulfate-15% polyacrylamide gel. Lane M contains molecular weight markers of which the mass is indicated on the left (in kDa). The other five lanes contain the ECPs that were purified from apoplastic fluids isolated two weeks after inoculation of susceptible tomato plants with race 5 of *C. fulvum*.

Identification of tomato lines and wild *Lycopersicon* plants that specifically respond with HR to each of the isolated ECPs

HR is a hallmark of resistance of tomato against *C. fulvum*, as has been reported for most introgressed resistance genes studied so far (De Wit, 1992). Therefore, it is expected that breeding lines preliminary selected for resistance against the fungus, potentially respond with HR after exposure to a particular matching fungal protein. We screened a collection of 28 genotypes originating from breeding programmes of several institutes. As a negative control we used the cultivar Moneymaker that does not contain any known gene for resistance against *C. fulvum*. Injection of AFs isolated from compatible interactions involving any *C. fulvum* strain tested so far in our laboratory has never been found to trigger a detectable HR in this cultivar. Each genotype was tested for its ability to respond with HR upon injection of each of the five purified ECPs. Besides the lines that displayed HR to ECP2 (Laugé *et al.*, 1998b), we identified two additional lines that responded specifically with HR to an ECP. Line #80 responded with HR to ECP3, and line #95 responded with HR to ECP5 (Figure 2). In both cases the injected part of the leaf became chlorotic and necrotic a few days after injection. None of the breeding lines responded with HR upon injection of ECP1 or ECP4.
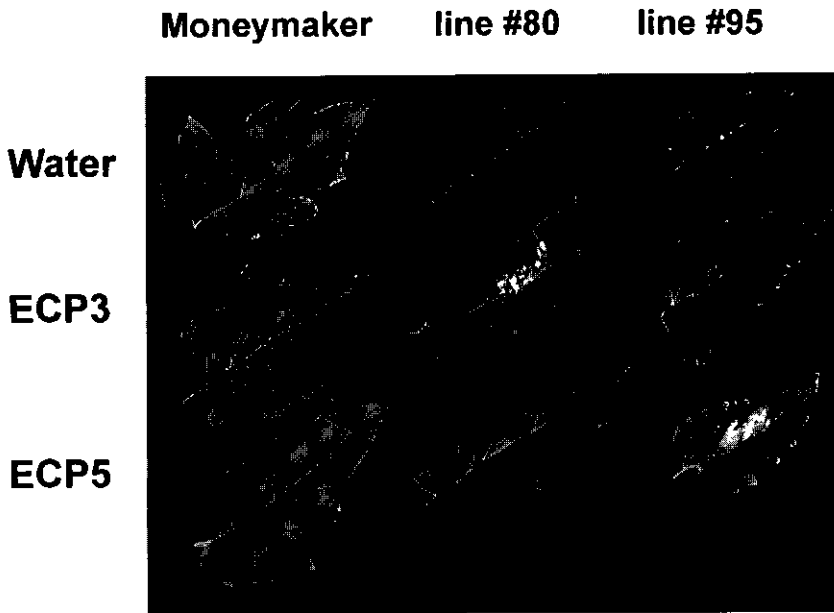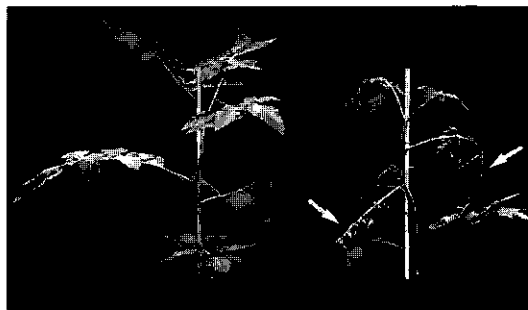

Figure 2. Hypersensitive response (HR) induced in tomato breeding lines upon injection with purified ECP3 or ECP5.
Two tomato breeding lines, #80 and #95, were identified that responded with HR to ECP3 and ECP5, respectively. Cultivar Moneymaker was used as a control. Three leaflets of each plant genotype were injected with either water, purified ECP3 or purified ECP5. Specific HR occurs in the leaflet of line #80 injected with ECP3, and in the leaflet of line #95 injected with ECP5. Photographs were taken three days after injection.

Most of the existing breeding lines displaying resistance against *C. fulvum* originate from currant tomato, *Lycopersicon pimpinellifolium*, a wild species that can be easily crossed with the closely related cultivated tomato, *L. esculentum*. We extended our search for plants responding with HR to ECP1 or ECP4, by screening a collection of 40 wild accessions of *L. pimpinellifolium* using recombinant potato virus (PVX) (Table 1). PVX is a potexvirus that causes systemic mild mosaic symptoms on tomato. It has previously been used as an expression system for the cDNAs of *Avr9*, *Avr4*, and *Ecp2* as transgenes in the genome of the virus. The resulting PVX::*Avr9*, PVX::*Avr4* and PVX::*Ecp2* recombinant derivatives caused a systemic HR on tomato genotypes containing the *Cf-9*, *Cf-4* and *Cf-ECP2* resistance genes, respectively (Hammond-Kosack *et al.*, 1995; Joosten *et al.*, 1997, Laugé *et al.*, 1998b). The *Ecp1* and *Ecp4* cDNAs were used in a similar way to construct PVX::*Ecp1* and PVX::*Ecp4* derivatives that allowed screening for HR on a large number of plants. From each accession, four plants were inoculated with PVX::*Ecp1* and four plants were inoculated with PVX::*Ecp4*. Seven to ten days after inoculation, the plants were examined for the development of systemic HR. In one accession, *Lp#18*, one plant was identified that showed systemic HR to PVX::*Ecp1*, whereas in three other accessions, *Lp#29*, *Lp#31* and *Lp#32*, all four plants showed systemic HR to PVX::*Ecp4* (Figure 3). The rescued ECP1-responding plant and one representative of the ECP4-responding plants were selfed. The resulting progenies were used to confirm the response of the genotypes to either ECP1 or ECP4, to study the specificity of this response, and to characterize its heritability. Twenty S1 offspring plants were inoculated either with a wild-type PVX, or any of the five PVX derivatives, PVX::*Avr4*, PVX::*Avr9*, PVX::*Ecp1*, PVX::*Ecp2* and PVX::*Ecp4*. The complete progeny of accession *Lp#18* and the complete progeny of accession *Lp#31* responded with HR to PVX::*Ecp1* and PVX::*Ecp4*, respectively. This confirmed the results of the initial screening and also shows that the specific recognition in each plant genotype is caused by a dominant trait. Injection of purified ECP1 and ECP4 in the same progeny plants showed the specific induction of HR (Figure 3).

Table 1. Systemic hypersensitive response induced in accessions of *Lycopersicon pimpinellifolium* upon inoculation with PVX::*Ecp*1 or PVX::*Ecp*4.

	PVX derivative	
	PVX::*Ecp*1	PVX::*Ecp*4
Lycopersicon pimpinellifolium		
*Lp#*1	-	-
*Lp#*2	-	-
*Lp#*3	-	-
*Lp#*4	-	-
*Lp#*5	-	-
*Lp#*6	-	-
*Lp#*7	-	-
*Lp#*8	-	-
*Lp#*9	-	-
*Lp#*10	-	-
*Lp#*11	-	-
*Lp#*12	-	-
*Lp#*13	-	-
*Lp#*14	-	-
*Lp#*15	-	-
*Lp#*16	-	-
*Lp#*17	-	-
*Lp#*18	1/4	-
*Lp#*19	-	-
*Lp#*20	-	-
*Lp#*21	-	-
*Lp#*22	-	-
*Lp#*23	-	-
*Lp#*24	-	-
*Lp#*25	-	-
*Lp#*26	-	-
*Lp#*27	-	-
*Lp#*28	-	-
*Lp#*29	-	4/4
*Lp#*30	-	-
*Lp#*31	-	4/4
*Lp#*32	-	4/4
*Lp#*33	-	-
*Lp#*34	-	-
*Lp#*35	-	-
*Lp#*36	-	-
*Lp#*37	-	-
*Lp#*38	-	-
*Lp#*39	-	-
*Lp#*40	-	-

S1 *Lp*#18/PVX wt   **S1 *Lp*#18/PVX::*Ecp*1**

S1 *Lp*#31/PVX wt   **S1 *Lp*#31/PVX::*Ecp*4**

ECP1   **ECP4**
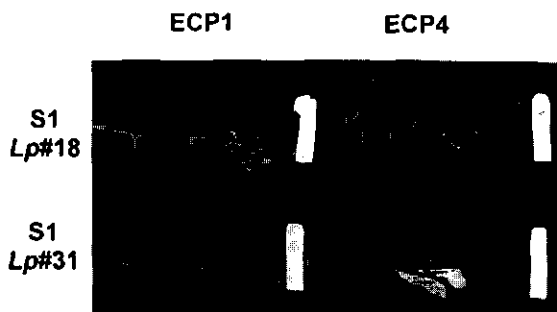
S1
***Lp*#18**

S1
***Lp*#31**

Figure 3. Hypersensitive response (HR) of accessions of *Lycopersicon pimpinellifolium* upon exposure to the ECP1 or the ECP4 proteins.

A) Identification of one plant from accession *Lp*#18 exhibiting HR to ECP1. While mild systemic mosaic symptoms are visible on the S1 offspring of the identified plant upon inoculation with PVX wild-type (wt), systemic HR (white arrows) develops in the same offspring upon inoculation with the recombinant PVX::*Ecp*1. Note the characteristic epinasty, which is followed by development of systemic HR. B) Identification of plants from accessions *Lp*#29, *Lp*#31, and *Lp*#32 exhibiting HR to ECP4. Shown is the S1 offspring from one *Lp*#31 mother plant. Mild mosaic symptoms are visible upon inoculation with PVX wild-type, but systemic HR (white arrows) develops on the same offspring upon inoculation with the recombinant PVX::*Ecp*4. Photographs A) and B) were taken two weeks after inoculation with the viruses. C) HR on the identified plants upon injection of the ECP1 and ECP4 proteins. ECP1 and ECP4 were injected in leaflets of S1 plants from the responding accessions, and consistent with the results of the PVX screening, specific HR develops in the injected area of the corresponding plants. Photographs were taken three days after injection of the ECPs.

Specific HR-associated recognition of ECP2 in a non-host plant confers resistance against an unrelated pathogen

C. fulvum has a narrow host range, which is restricted to the *Lycopersicon* genus (Bond, 1938). We took advantage of the broad host range of PVX, that comprises many solanaceous genera, to test whether these non-host plants of *C. fulvum* can also respond with HR to any of the isolated AVR or ECP proteins. A collection of 48 accessions from wild species belonging to the genera *Nicotiana* and *Petunia* (Table 2) were inoculated with PVX::*Avr4*, PVX::*Avr9*, PVX::*Ecp*1, PVX::*Ecp*2 and PVX::*Ecp*4 derivatives. From each accession, eight plants were inoculated with each of the five PVX derivatives, as well as with the wild-type PVX as a control. Two accessions of *N. paniculata* were identified from which all plants responded with HR to ECP2 (Figure 4). The other PVX derivatives did not induce HR on any of these accessions. ECP2-responding plants were selfed and progenies were inoculated with the wild-type PVX or with the PVX::*Ecp*2 derivative. The progeny plants displayed mosaic symptoms to wild-type PVX and HR to PVX::*Ecp*2, which confirmed that the response is due to exposure to ECP2.

Different from the *Cf-ECP2* tomato genotype inoculated with PVX::*Ecp*2, or any *Lycopersicon* plants showing systemic HR to any PVX derivative, the *N. paniculata* plants exhibited local HR confined to the inoculated leaves rather than systemic HR (Figure 4). This indicates that the ECP2-mediated HR arrests systemic spread of PVX::*Ecp*2. Thus PVX expressing *Ecp*2 has become avirulent on these two *N. paniculata* accessions on which it is normally virulent.

Table 2. Systemic hypersensitive response induced in accessions of *Nicotiana* and *Petunia* species upon inoculation with PVX::*Avr4*, PVX::*Avr9*, PVX::*Ecp1*, PVX::*Ecp2* or PVX::*Ecp4*.

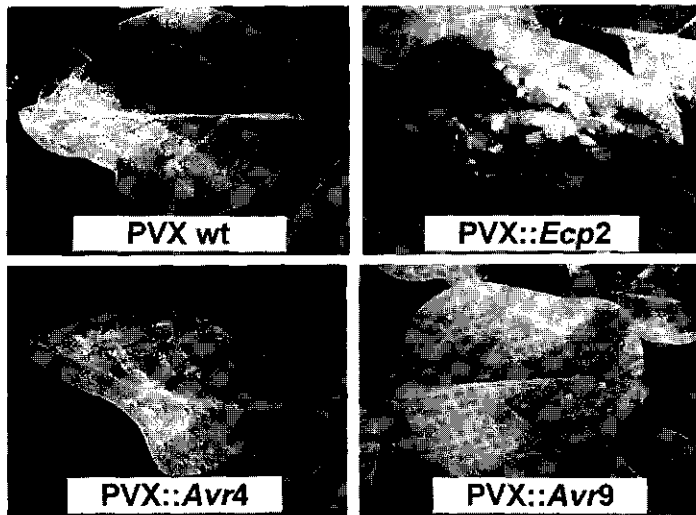|	PVX derivative				
	PVX::*Avr4*	PVX::*Avr9*	PVX::*Ecp1*	PVX::*Ecp2*	PVX::*Ecp4*
Nicotiana spp.					
N#1	-	-	-	-	-
N#2	-	-	-	-	-
N#3	-	-	-	-	-
N#4	-	-	-	-	-
N#5	-	-	-	-	-
N#6	-	-	-	-	-
N#7	-	-	-	-	-
N#8	-	-	-	-	-
N#9	-	-	-	-	-
N#10	-	-	-	-	-
N#11	-	-	-	-	-
N#12	-	-	-	-	-
N#13	-	-	-	-	-
N#14	-	-	-	-	-
N#15	-	-	-	-	-
N#16	-	-	-	-	-
N#17	-	-	-	-	-
N#18	-	-	-	-	-
N#19	-	-	-	-	-
N#20	-	-	-	-	-
N#21	-	-	-	-	-
N#22	-	-	-	-	-
N#23	-	-	-	-	-
N#24	-	-	-	-	-
N#25	-	-	-	-	-
N#26	-	-	-	-	-
N#27	-	-	-	-	-
N#28	-	-	-	-	-
N#29	-	-	-	-	-
N#30	-	-	-	-	-
N#31	-	-	-	-	-
N#32	-	-	-	8/8	-
N#33	-	-	-	-	-
N#34	-	-	-	8/8	-
N#35	-	-	-	-	-
N#36	-	-	-	-	-
N#37	-	-	-	-	-
N#38	-	-	-	-	-
N#39	-	-	-	-	-
N#40	-	-	-	-	-
N#41	-	-	-	-	-
N#42	-	-	-	-	-
N#43	-	-	-	-	-
N#44	-	-	-	-	-
Petunia spp.					
P#1	-	-	-	-	-
P#2	-	-	-	-	-
P#3	-	-	-	-	-
P#4	-	-	-	-	-

Figure 4. Identification of accessions of two *Nicotiana paniculata* exhibiting specific HR upon exposure to ECP2.

Plants of accession *N#34* are shown as representatives. One week after inoculation with the viruses, only inoculation damage is visible on the leaves of the plants challenged with PVX wild-type, PVX::*Avr4* and PVX::*Avr9*, whereas local lesions are visible on the leaves of plants challenged with PVX::*Ecp2*. Two weeks after inoculation, mosaic symptoms develop systemically on plants inoculated with PVX wild-type, PVX::*Avr4* and PVX::*Avr9*, whereas no symptoms are detectable on systemic leaves of the plants inoculated with PVX::*Ecp2*.

DISCUSSION

Due to the strict extracellular growth of the fungus, the gene-for-gene relationships in the *C. fulvum*-tomato interaction have been hypothesized to result from recognition of extracellular proteins produced by the fungus. The identification of the avirulence factors AVR4 and AVR9 that specifically induce HR on tomato genotypes containing the matching resistance genes, *Cf-4* and *Cf-9* respectively, confirmed that specific induction of HR by fungal proteinaceous elicitors is the basis of resistance against *C. fulvum* in the *Lycopersicon* genus. The discovery of a new resistance gene, based on recognition of ECP2 (Laugé *et al.*, 1998b), and the functionality of some *Cf-9* homologues responding to putative yet unidentified fungal elicitors (Parniske *et al.*, 1997; Laugé *et al.*, 1998a; Takken *et al.*, 1998),

allowed us to assume that specific HR-associated recognition of proteins of *C. fulvum* is probably a widely occurring phenomenon. Here we have demonstrated that five fungal proteins act as specific elicitors of HR on individuals of particular lines of tomato, wild *Lycopersicon* accessions and wild *Nicotiana* accessions. All responding tomato lines identified so far are resistant against *C. fulvum* and, although not yet proven, we expect that resistance relies on the HR-associated recognition of the characterized fungal proteins. For the *Nicotiana* individuals that are non-hosts of *C. fulvum*, we have demonstrated that HR-induction by PVX harbouring *Ecp2* confers resistance to that virus, indicating that HR induced by a heterologous protein can provide resistance against a heterologous pathogen. Thus, HR is a widely occurring defense response in plants which is effective against many unrelated pathogens, as has already been demonstrated upon transfer of a particular resistance gene to the genome of a different plant (Whitham *et al.*, 1996; Kamoun *et al.*, in press).

During the past four years, many *R* genes conferring resistance against various types of pathogens (e.g. viruses, bacteria, fungi, nematodes and aphids) have been cloned from Arabidopsis, tomato, tobacco, sugarbeet, rice and flax (Hammond-Kosack and Jones, 1997; De Wit, 1997). They are members of multigene families, subdivided into different classes based on common motifs such as leucine-rich-repeats, nucleotide-binding sites, kinase domains and domains homologous to mammalian receptors. The occurrence of these related families of *R* genes and their homologues in botanically unrelated plant species, suggests the existence of a broad range surveillance system in plants, providing protection against putative pathogens. From tomato, four *R* genes, *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*, conferring resistance to *C. fulvum*, have been cloned (Jones *et al.*, 1994; Dixon *et al.*, 1996; Thomas *et al.*, 1997; Dixon *et al.*, in press). Although these *R* genes confer resistance through recognition of unrelated fungal proteins, the *R*-gene products share extensive structure and sequence similarities. Many *Cf* homologues are present and organised in clusters throughout the tomato genome (Parniske *et al.*, 1997). Sequence analyses of the *Cf-4/9* locus in several haplotypes has revealed that sequence duplication, gene recombination, gene conversion and diversifying selection in the solvent-exposed part of the leucine-rich repeats has occurred (Parniske *et al.*, 1997). These molecular mechanisms all facilitate diversification of *Cf* homologues resulting in the generation of new recognition specificities. Here, we have shown that distinct recognition specificities occur abundantly within the *Lycopersicon* genus. It remains to be determined whether the genes responsible for HR-associated recognition of each ECP are *Cf* homologues. However, the identified tomato lines and *Lycopersicon* individuals show that the *Lycopersicon* genus can potentially respond with HR to any *C. fulvum* protein.

Our finding that some *Nicotiana* individuals display HR upon exposure to ECP2, suggests that recognition resulting in HR does not only occur in the host plant(s) of the pathogen. Selection for maintenance of recognition specificities in the population might take place under selection pressure exerted by a pathogen. In this way, the plant would co-evolve with the pathogen by accumulating resistances operating through recognition of molecules of microbial origin as had been the case for *Lycopersicon*, where there exist individuals

responding to every ECP protein tested so far. However, we cannot exclude that a yet unidentified pathogen of tobacco produces an elicitor that shares structural homology with ECP2. Screening of larger collections of *Nicotiana* species may show that other AVR or ECP proteins can induce HR in additional individuals. Interestingly, HR induced by ECP2 in *N. paniculata* individuals proved to provide resistance against PVX. Furthermore, the resistance response resembles the natural resistance conferred by the *N* gene in *N. glutinosa* against the unrelated tobacco mosaic virus (Whitham *et al.*, 1994). This might indicate that the ECP2-mediated resistance against PVX recruits similar HR-related signal transduction pathway(s) as the *N* gene.

Altogether, our data and the data on the *Cf* genes support the existence of a versatile recognition system for non-self proteins in populations of plant species that accounts for the various gene-for-gene relationships described for many host-pathogen combinations. We propose that multiple recognition specificities conferred by sets of homologues of the reported *R* genes are carried by individuals within one plant species. Among those genes, the functional ones that recognize foreign proteins and activate a signal transduction pathway leading to HR would become *R* genes. The variety of resistance specificities present in one plant corresponds to the variety of foreign proteins that its *R* gene homologues can recognize and respond to with HR. In this way, between a given host individual and a given pathogenic strain three levels of interactions are possible: i) none of the proteins produced by the pathogen is recognized: no HR will occur and the interaction will be compatible; ii) one or several proteins are recognized by the host, while none induces an early and strong HR: the interaction will be partially compatible; iii) at least one protein is recognized by the host which induces an early and strong HR: the interaction is incompatible. The latter two types of interactions will lead to gene-for-gene relationships between the plant and the pathogen, of which mainly the *R* genes with major effects will be used in resistance breeding programs.

For plants, an HR-associated protection system with numerous recognition specificities generated at random and scattered among individuals within a population, would probably represent the best evolutionary solution to withstand infection by pathogens. Different from animals, plants lack internal streams where circulation of cells can occur which is the basis of an efficient somatic surveillance system such as the mammalian immune system. Within a plant species, surveillance specificities are carried by individuals. Given a distribution of the specificities as discussed above, for any outbreak of a severe pathogen, there would always be a few individuals that can recognize some of the proteins produced by the pathogen. In nature, where many pathogens can occur on one particular plant species, the combination of these individuals would ensure the survival of the species.

Our model is supported by the experimental data showing that certain resistance genes against different types of pathogens map at similar positions in the genome of several plants. Also the genetic structure and mechanism of diversification of the resistance gene clusters studied so far (Dangl, 1992; Parniske *et al.*, 1997; Song *et al.*, 1997) have been noticed to be highly reminiscent of the structure and function of the mammalian major

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Chapter 6

Additional resistance gene(s) against *Cladosporium fulvum* present on the *Cf-9* introgression segment are associated with strong PR protein accumulation

Additional Resistance Gene(s) Against *Cladosporium fulvum* Present on the *Cf-9* Introgression Segment Are Associated with Strong PR Protein Accumulation

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The existence of a gene or genes conferring weak resistance against the fungal tomato pathogen *Cladosporium fulvum*, in addition to the *Cf-9* resistance gene, present on the *Lycopersicon pimpinellifolium Cf-9* segment introgressed into *L. esculentum*, was demonstrated with strains of *C. fulvum* lacking a functional *Avr9* avirulence gene and tomato genotypes lacking a functional *Cf-9* gene, respectively. Two mutant strains, obtained by disruption of *Avr9* in race 4 and race 5 of *C. fulvum*, do not trigger the hypersensitive response–mediated resistance on MM-Cf9 genotypes that is normally induced after recognition of the AVR9 elicitor. However, when these strains are inoculated onto MM-Cf0 and MM-Cf9 genotypes, adult MM-Cf9 plants still show weak resistance. This resistance is not related to the *Cf-9* gene, as ethyl methanesulfonate (EMS)–generated *Cf-9* mutants retained weak resistance. Growth of the fungus in the leaf mesophyll is strongly inhibited, whereas re-emergence of fungal mycelium and conidiation are poor. Strong accumulation of pathogenesis-related proteins and early leaf chlorosis are associated with this phenotype of weak resistance. A search among natural strains lacking the *Avr9* gene revealed that one strain is able to overcome this weak resistance. Possible mechanisms underlying this weak resistance are discussed. The presence of the additional weak resistance gene(s) could explain why the resistance of Cf9 genotypes has not been overcome so far in practice.

The imperfect fungus *Cladosporium fulvum* Cooke causes leaf mold disease on tomato (*Lycopersicon esculentum* Mill.). Under high humidity conditions, conidia of the fungus present at the lower side of tomato leaves germinate, form runner hyphae, and grow over the surface until open stomata are encountered. After penetration through stomata, the fungus develops biotrophically in the apoplastic space of the mesophyll, without producing specialized feeding structures. After 2 to 3 weeks, the fungus emerges from the leaf tissue through stomata and conidiophores develop that produce large amounts of conidia (De Wit 1977).

Resistance toward *C. fulvum* has been found in several wild species of the genus *Lycopersicon*. Resistance is a monogenic dominant character and provides either an immune type of resistance, which is based on a hypersensitive response (HR) allowing no fungal colonization, as has been described for the *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* resistance genes, or a weak type of resistance in which residual colonization takes place, such as for the *Cf-1* and *Cf-3* resistance genes (Stevens and Rick 1988). Genetic variation of the fungus has resulted in the appearance of new physiologic races that could overcome resistance genes introgressed into commercial tomato cultivars (Day 1956, 1957). New resistance genes were found in wild *Lycopersicon* spp. (Kanwar et al. 1980; Stamova and Yordanov 1977, 1978a, 1978b). So far all resistance genes used in breeding programs act independently. Additional *C. fulvum* resistance genes have been described; however, allelism tests have not yet been conducted systematically to prove that these genes are distinct from already characterized resistance genes. Apparently, many independent resistance genes toward *C. fulvum* occur in nature.

Four resistance genes, *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9*, introgressed from three different *Lycopersicon* spp., have been investigated in detail. *Cf-4* and *Cf-9* are closely linked on chromosome 1, while *Cf-2* and *Cf-5* are closely linked on chromosome 6 (Jones et al. 1993). Furthermore, *Cf-1*, a weak resistance gene, maps near the *Cf-4/Cf-9* genes (Langford 1937; Kerr and Bailey 1964). Thus, a number of the *Cf* genes appear to be clustered.

Molecular analysis of proteins secreted by *C. fulvum* during its growth in the apoplast of tomato leaves led to the identification of the race-specific elicitors AVR9 and AVR4 (Scholtens-Toma and De Wit 1988; Joosten et al. 1994). These proteinaceous elicitors govern recognition of the fungus by resistant plants carrying the resistance genes *Cf-9* and *Cf-4*, respectively. The two encoding genes, *Avr9* and *Avr4*, and the matching resistance genes *Cf-9* and *Cf-4*, respectively, comply with the gene-for-gene hypothesis (Van den Ackerveken et al. 1992; Joosten et al. 1994).

Cloning of the four *Cf* genes revealed that they are highly homologous (Jones et al. 1994; Dixon et al. 1996; Hammond-Kosack and Jones 1997). They encode putative extracellular glycoproteins containing leucine-rich repeats (LRRs), with a transmembrane domain and a short C-terminal cytoplasmic

tail. They differ mainly in the number of LRRs and certain amino acids in the LRRs (Jones et al. 1996). Southern analyses of the tomato genome demonstrated that these genes constitute gene families (Jones et al. 1996) and sequence analyses showed that *Cf* genes are organized in clusters of homologues (Hammond-Kosack and Jones 1997). The *Cf-9* gene was shown to be part of a region containing five homologous genes, among which one is the functional *Cf-9* gene. This finding suggested that homologues of the *Cf* genes might have the capacity to recognize yet unidentified elicitors of *C. fulvum*.

To test whether *Cf* resistance genes, in addition to the functional *Cf-9* gene, are present on the *Cf-9* introgression fragment from *L. pimpinellifolium* we used two transgenic fungal strains in which the *Avr9* gene has been disrupted (Marmeisse et al. 1993). In the absence of the AVR9 elicitor, presence of the functional *Cf-9* gene would no longer result in recognition of these strains, but *Cf-9* homologues of the cluster or other genes at the locus might still do so. Here we report on virulence of *Avr9* mutants on near-isogenic lines of the tomato cv. Moneymaker, either lacking the *Cf-9* introgression segment (MM-Cf0), or containing the *Cf-9* introgression segment with (MM-Cf9) or without (MM-Cf9/*Cf-9* mutant) a functional *Cf-9* gene. Results indicate that additional *Cf* resistance gene(s) are present on the *Cf-9* introgression segment, whose function is *Avr9*/*Cf-9*–independent.
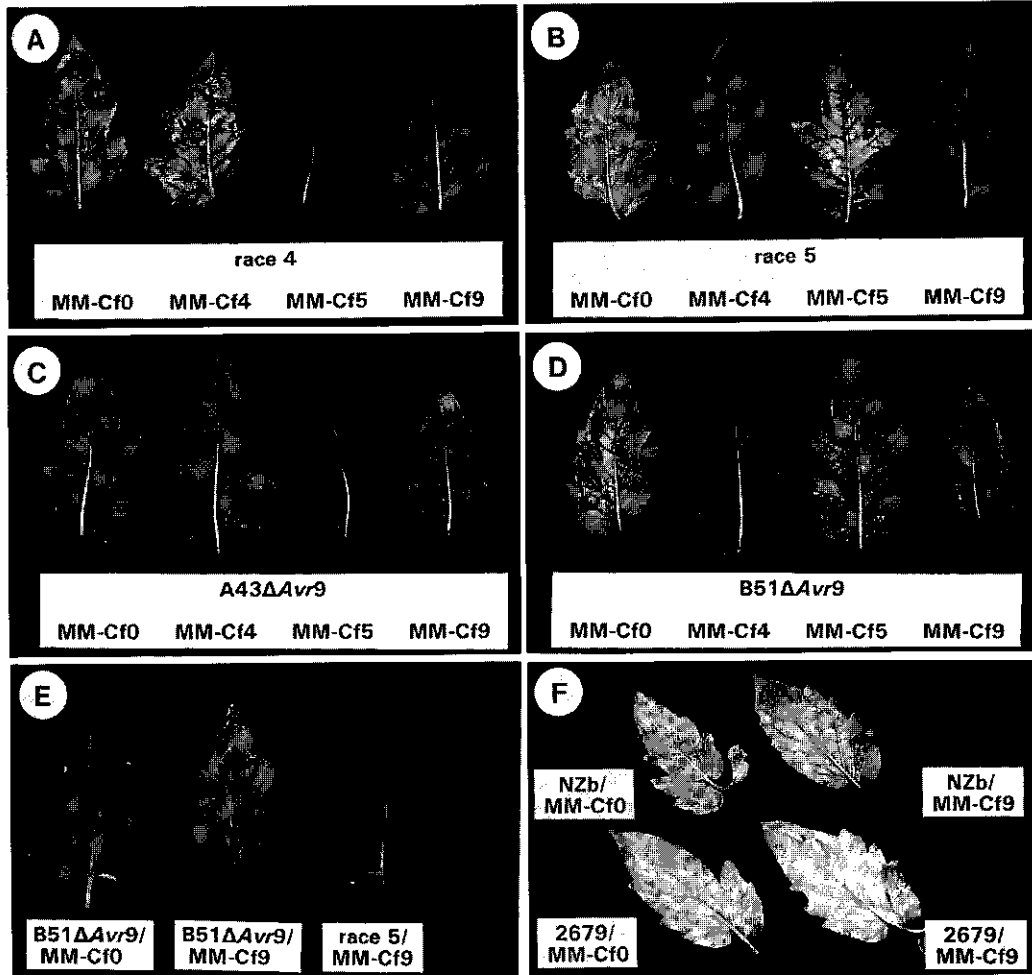
Fig. 1. Disease symptoms present at the lower side of leaflets of near-isogenic lines of the tomato cv. Moneymaker carrying different *Cf* resistance genes, 3 weeks post inoculation with (**A**) race 4, (**B**) race 5, (**C**) *Avr9*-disrupted strain A43ΔΔvr9, and (**D**) *Avr9*-disrupted strain B51ΔΔvr9. Emerging mycelium forms white and brown patches that will eventually release conidia. **E**, Disease symptoms present at the upper side of leaflets 2 weeks post inoculation; from left to right, MM-Cf0 plants inoculated with the strain B51ΔΔvr9, MM-Cf9 plants inoculated with B51ΔΔvr9, and MM-Cf9 plants inoculated with race 5. **F**, Symptoms at the lower side of leaflets of MM-Cf0 and MM-Cf9 plants 3 weeks post inoculation with the natural *Avr9* mutants NZb and 2679.

RESULTS

A weak resistance, present on the *Cf-9* introgression segment, is independent of AVR9 recognition.

Fungal strains, race 4, race 5, and the two corresponding isogenic strains obtained after disruption of the *Avr9* gene, A43ΔΔ*Avr9*, and B51ΔΔ*Avr9*, were inoculated on MM-Cf0, MM-Cf4, MM-Cf5, and MM-Cf9 tomato genotypes.

After colonization of the mesophyll, fungal mycelium emerges through stomata at the lower side of the leaf, and the density of the white and brown mycelium patches is a measure of disease severity. Symptoms developed 3 weeks post inoculation are presented in Figure 1A, B, C, and D. From Figure 1 it is clear that the tomato genotype without a known resistance gene (MM-Cf0) is fully susceptible to all races of *C. fulvum*, irrespective of whether the *Avr9* gene is present or absent. Similarly, the MM-Cf4 and MM-Cf5 tomato genotypes are fully susceptible to race 4 and race 5, respectively, as well as to the corresponding isogenic *Avr9*-disrupted strains, A43ΔΔ*Avr9* and B51ΔΔ*Avr9*, respectively. The severity of the disease symptoms caused by these strains on MM-Cf4 and MM-Cf5 is not different from the severity of the disease symptoms caused on MM-Cf0. However, the severity of the disease symptoms caused by strains A43ΔΔ*Avr9* and B51ΔΔ*Avr9* on MM-Cf0 and MM-Cf9 is different. Strains lacking the *Avr9* gene, generated in two independent fungal genetic backgrounds, cause less disease symptoms on MM-Cf9 than on MM-Cf0. These observations indicate that a second weak type of resistance, in addition to the *Avr9*/*Cf-9*-mediated HR-type of resistance, is present on the segment introgressed from *L. pimpinellifolium* into MM-Cf9 plants. This resistance is clearly different from the full resistance induced by the recognition of AVR9 mediated by the *Cf-9* gene, as disease symptoms are still present. Differences in the severity of the disease symptoms were reproducible between the plant individuals within the same inoculation experiment and between repeated experiments. Interestingly, there is no difference in the severity of the disease symptoms between the MM-Cf0 and the MM-Cf4 genotypes upon inoculation with race 4, nor between the MM-Cf0 and the MM-Cf5 genotypes upon inoculation with race 5. The latter observation suggests that, at least with the two fungal strains tested, no additional weak resistance is conferred by the MM-Cf4 and MM-Cf5 genotypes that contain introgression segments originating from *L. hirsutum* and *L. esculentum* var. *cerasiforme*, respectively.

The weak resistance is independent of the presence of a functional *Cf-9* gene.

The weak resistance is independent of *Avr9*, one partner of the *Avr9*/*Cf-9* gene pair. We checked whether it is also independent of the other partner, the *Cf-9* resistance gene. We used MM-Cf9/*Cf-9* mutant genotypes that were obtained by ethyl methanesulfonate (EMS) treatment of MM-Cf9 plants (Hammond-Kosack et al. 1994). The resulting plants carry the *L. pimpinellifolium* introgression segment lacking a functional *Cf-9* gene.

Upon inoculation with the two *Avr9* mutants, the *Cf-9* mutants show disease symptoms that are similar to those observed on MM-Cf9 plants, indicating that the weak resistance factor is present in the *Cf-9* mutants (data not shown). Therefore, this weak resistance is independent of the *Cf-9* resistance

gene and does not relate to some secondary function of *Cf-9*. As expected, it was observed that the *Cf-9* mutants, which are no longer responsive to AVR9, exhibit weak resistance toward the wild-type strains, races 4 and 5.

The weak resistance results in inhibition of fungal growth and conidiation.
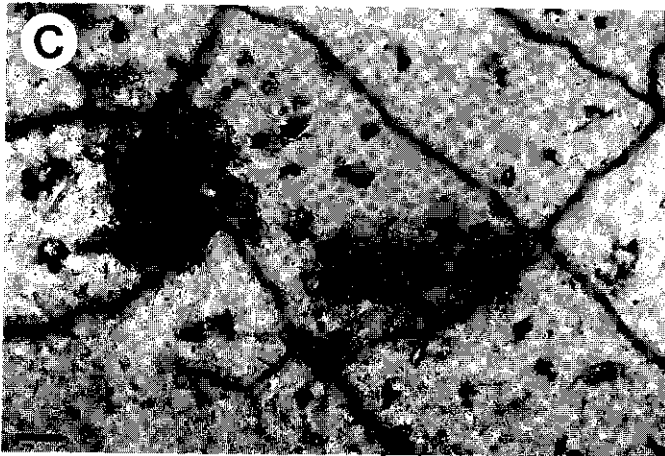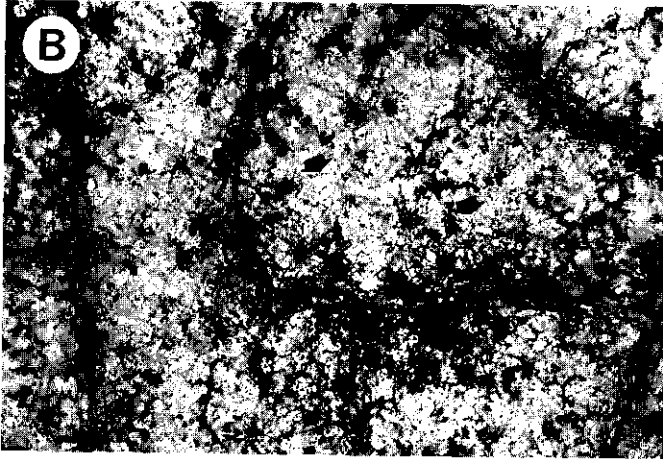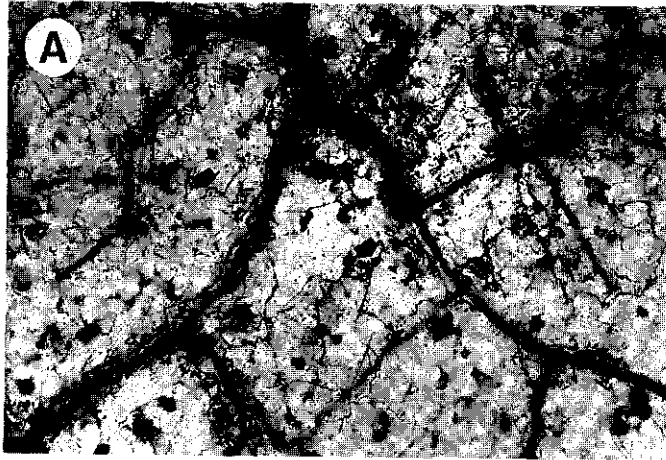
Fungal growth inside the leaf was studied by light microscopy 20 days post inoculation (Fig. 2). Leaves of MM-Cf9 plants are poorly colonized by the B51ΔΔ*Avr9* strain (Fig. 2A), when compared with the colonization of the MM-Cf0 plants (Fig. 2B). Furthermore, the mycelium appears swollen in leaves from MM-Cf9 plants, which is not observed in leaves from MM-Cf0 plants (Fig. 2A, see arrows). However, inhibition of fungal growth does not reach the level exhibited by MM-Cf9 plants when inoculated with the natural AVR9-producing race 5, where hyphae are eventually contained within the area of cells showing HR (Fig. 2C). Similar results were obtained for the A43ΔΔ*Avr9* and race 4 strains.

To extend the microscopical observations, we determined the accumulation of two in planta–secreted proteins of *C. fulvum*, ECP1 (Joosten and De Wit 1988) and ECP2 (Wubben et al. 1994), by immunodetection. These proteins can be used as quantitative markers of the fungal biomass in planta (Laugé et al. 1997). Proteins present in apoplastic fluids (AFs) were subjected to polyacrylamide gel electrophoresis (PAGE) and Western blots (immunoblots) were probed with antibodies raised against the two proteins. The results obtained with the ECP1 antibodies are shown in Figure 3. MM-Cf0 plants inoculated with either the wild-type strain or with the corresponding *Avr9*-disrupted strain show accumulation of ECP1 to similar levels (compare lanes 1 and 3, and 5 and 7). However, AFs from MM-Cf9 plants inoculated with the A43ΔΔ*Avr9* or B51ΔΔ*Avr9* strains show much less accumulation of ECP1 when compared with MM-Cf0 plants inoculated with these strains (compare lanes 3 and 4, and 7 and 8). No accumulation of ECP1 was detected in AFs from MM-Cf9 plants inoculated with the natural AVR9-producing strains (lanes 2 and 6). Similar results were obtained upon immunodetection of ECP2 (results not shown). These results indicate that the weak resistance results in inhibition of fungal growth in the leaf mesophyll.

Macroscopic observations showed that, associated with less severe disease symptoms, both the A43ΔΔ*Avr9* and B51ΔΔ*Avr9* strains exhibit reduced conidiation on MM-Cf9 plants when compared with MM-Cf0 plants (Fig. 1C and D).

The weak resistance response is associated with accumulation of high levels of PR proteins and extensive leaf chlorosis.

We analyzed whether the observed levels of resistance are associated with different levels of pathogenesis-related (PR) protein accumulation. AFs obtained after inoculation of MM-Cf0 and MM-Cf9 plants, either with the strains race 4 and race 5 or with the A43ΔΔ*Avr9* and B51ΔΔ*Avr9* strains, were analyzed by sodium dodecyl sulfate (SDS)–PAGE. Samples were harvested every 2 days between 12 and 20 days post inoculation. Similar patterns were obtained for each time point and Figure 4 shows the protein profiles of the various AFs 12 days post inoculation. The highest levels of PR protein accumulation are observed in MM-Cf9 geno-

types inoculated with the A43ΔΔ*Avr9* and B51ΔΔ*Avr9* strains (lanes 5 and 9). In particular, P69 (Vera and Conejero 1988), β-1,3-glucanases (Joosten and De Wit 1989), and the 15-kDa P14 protein family (Joosten et al. 1990) accumulate to high levels. There is also accumulation of PR proteins in both the fully compatible (lanes 2, 4, 6, and 8) and incompatible (lanes 3 and 7) interactions, but much less than in the interactions in which the plant displays weak resistance. The accumulation of the various families of PR proteins was confirmed by Western blot analysis (results not shown).

Inoculation of the A43ΔΔ*Avr9* or B51ΔΔ*Avr9* strains on MM-Cf9 plants led to early and extensive chlorosis, easily detectable from the upper side of the leaf, as illustrated with MM-Cf9 plants inoculated with the B51ΔΔ*Avr9* strain in Figure 1E.

One natural strain of *C. fulvum* that overcomes the *Cf-9*–mediated resistance can also overcome the weak resistance.

We were interested to test whether natural strains of *C. fulvum* that overcome the *Cf-9*–mediated resistance are unable to overcome the weak resistance present on the *Cf-9* introgression segment, or whether some strains would exist that are able to overcome both resistances. We tested the few known strains that lack the *Avr9* gene from different geographical origins and inoculated them onto MM-Cf0 and MM-Cf9 plants. Based on symptom development and accumulation of ECP1 and ECP2 in planta, we found that the most recently isolated strain, strain 2679, caused disease symptoms on MM-Cf9 plants as severely as on MM-Cf0 plants (Figs. 1F, 5A). Consistently, the level of PR protein accumulation did not differ between MM-Cf9 plants and MM-Cf0 plants when inocu-

Fig. 2. Colonization of the leaf mesophyll, 3 weeks post inoculation of (A) MM-Cf9 plants with B51ΔΔ*Avr9*, (B) MM-Cf0 plants inoculated with race 5, (C) MM-Cf9 plants inoculated with race 5. Arrows point to fungal mycelium. A few hyphae are present in A; the mesophyll is fully colonized by mycelium in **B**, and fungal growth is arrested in C. Bar = 100 µm.
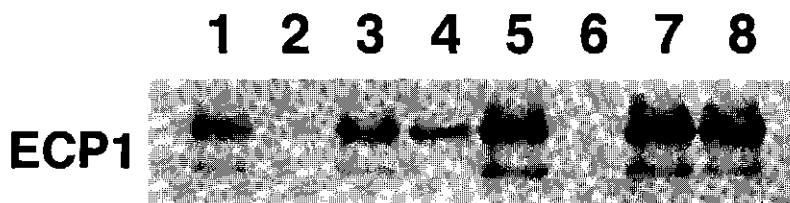
1 2 3 4 5 6 7 8

ECP1

Fig. 3. Immunodetection of the fungal, in planta–secreted ECP1 protein with 5 µl of apoplastic fluids (AFs) obtained 20 days post inoculation from the following interactions: lane 1, MM-Cf0 plant inoculated with race 4; lane 2, MM-Cf9 plant inoculated with race 4; lane 3, MM-Cf0 plant inoculated with A43ΔΔ*Avr9*; lane 4, MM-Cf9 plant inoculated with A43ΔΔ*Avr9*; lane 5, MM-Cf0 plant inoculated with race 5; lane 6, MM-Cf9 plant inoculated with race 5; lane 7, MM-Cf0 plant inoculated with B51ΔΔ*Avr9*; lane 8, MM-Cf9 plant inoculated with B51ΔΔ*Avr9*. Under native high-pH polyacrylamide gel electrophoresis conditions, ECP1 gives a double band most probably due to the presence of two differently processed forms of ECP1 in AF.
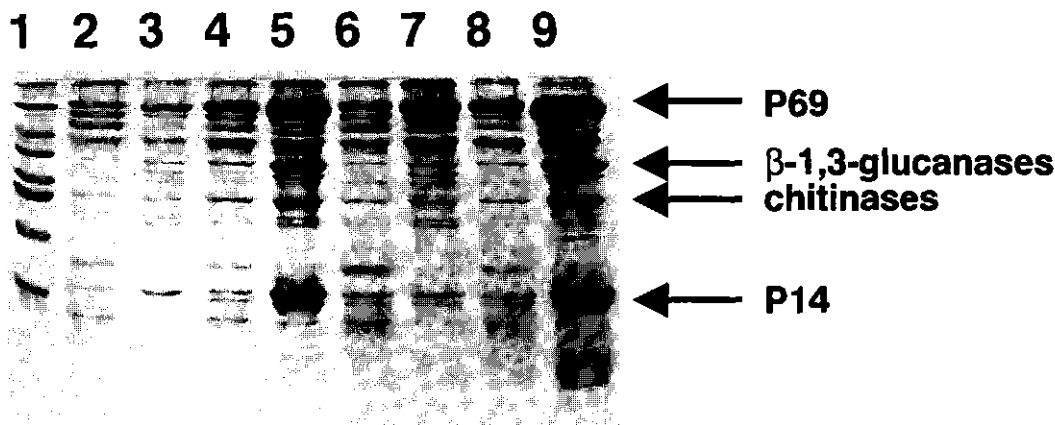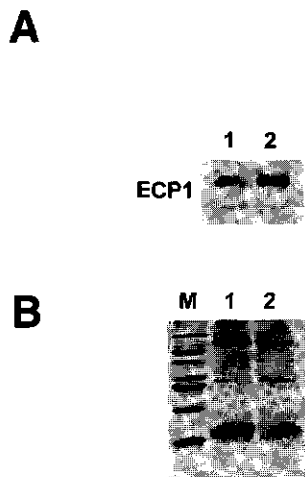
1 2 3 4 5 6 7 8 9

← **P69**

← **β-1,3-glucanases**
← **chitinases**

← **P14**

Fig. 4. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 100 µl of apoplastic fluid isolated from leaflets 12 days post inoculation. Lane 1, molecular weight marker; lane 2, MM-Cf0 plant inoculated with race 4; lane 3, MM-Cf9 plant inoculated with race 4; lane 4, MM-Cf0 plant inoculated with A43ΔΔ*Avr9*; lane 5, MM-Cf9 plant inoculated with A43ΔΔ*Avr9*; lane 6, MM-Cf0 plant inoculated with race 5; lane 7, MM-Cf9 plant inoculated with race 5; lane 8, MM-Cf0 plant inoculated with B51ΔΔ*Avr9*; lane 9, MM-Cf9 plant inoculated with B51ΔΔ*Avr9*. Classes of the most abundant pathogenesis-related proteins that accumulate in the apoplast are marked by an arrow (P69, β-1,3-glucanases, chitinases, and P14).

lated with strain 2679 (Fig. 5B). All other strains tested showed the same difference in disease symptoms on MM-Cf9 and on MM-Cf0 plants as was observed for the A43ΔAvr9 and B51ΔAvr9 mutants. This is illustrated for the strain NZb (Fig. 1F). Similarly, these strains triggered a higher level of PR protein accumulation upon inoculation on MM-Cf9 plants when compared with inoculation on MM-Cf0 plants (data not shown). Thus, in addition to overcoming the Cf-9-mediated resistance, the strain 2679 is able to overcome the weak resistance.

DISCUSSION

Here we present evidence for the existence of a gene or genes conferring weak resistance to C. fulvum in tomato, in addition to the Cf-9 resistance gene, on the Cf-9-containing segment introgressed from L. pimpinellifolium PI126933. The weak resistance, which is independent of the production of AVR9 by the invading fungus and of the presence of a functional Cf-9 gene, is associated with reduced colonization of the tomato leaf, reduction of conidia production, and strong accumulation of PR proteins in the apoplast of the infected leaves. A survey of Avr9 mutants occurring in nature identified one strain that overcomes the weak resistance.

A

1　2

ECP1

B

M　1　2

The two Avr9-disrupted strains that were constructed by Marmeisse et al. (1993) were initially inoculated on 2-week-old MM-Cf9 seedlings and it was shown that the strains could overcome the Cf-9-mediated resistance (Marmeisse et al. 1993). However, seedlings are not suitable to detect quantitative differences in virulence, as we have previously reported for mutant strains of C. fulvum lacking either Ecp1 or Ecp2 (Laugé et al. 1997). Decrease in virulence was apparent upon inoculation of 6-week-old plants. Similarly, studies with the EMS Cf-9 mutants did not reveal weak resistance, as they were performed on cotyledons of seedlings (Hammond-Kosack et al. 1994). However, the authors reported increased necrosis near vascular tissue, which they suggested is due to the action of an additional weak resistance gene.

When Avr9 mutants are inoculated on MM-Cf0 and MM-Cf9 plants, it is clear that the phenotype is determined by a plant trait and not by a fungal trait. In our inoculation tests we did not detect any difference in virulence between the race 4 and A43ΔAvr9 strains, or between the race 5 and B51ΔAvr9 strains on MM-Cf0, suggesting that Avr9 has no or a minor function in virulence of C. fulvum. Alternatively, it might be that the Avr9 function is complemented by other genes in the genetic backgrounds of races 4 and 5.

It has been shown that several of the Cf genes show epistasis for the resistance phenotype (Hammond-Kosack and Jones 1994). Therefore, it is conceivable that when several independent resistance genes against a certain strain of C. fulvum are originally present in one plant genotype, the strongest one will behave epistatically and mask the action of the weaker one(s). In order to reveal the presence of the weak gene(s), it is necessary to abolish the activity of the strong gene, either by deleting the avirulence gene encoding the matching eliciting compound in the particular fungal strain, or by inactivating the strong resistance gene itself in the plant. In our study the use of Avr9-disrupted strains and of EMS Cf-9 mutants allowed the discovery of the weak resistance gene(s). Thus, the outcome of the interaction between strains of C. fulvum and MM-Cf9 plants appears to be the result of the activity of several independent resistances, as illustrated in Table 1.

Table 1. Resistance levels of tomato genotypes challenged by various strains of Cladosporium fulvum

C. fulvum strains	MM-Cf0[a]	MM-Cf9[b]	MM-Cf9/ Cf-9 mutant[c]
Avr9+ wild types			
Race 4	S[d]	R[e]	WR[f]
Race 5	S	R	WR
Avr9 mutants (transgenic)			
A43ΔAvr9	S	WR	WR
B51ΔAvr9	S	WR	WR
Avr9 mutants (natural)			
NZb	S	WR	ND[g]
2679	S	S	ND

[a] Genotype lacking the Cf-9 introgression segment.
[b] Genotype containing the Cf-9 introgression segment.
[c] Genotype containing the Cf-9 introgression segment, without a functional Cf-9 resistance gene.
[d] Susceptible.
[e] Highly resistant.
[f] Weakly resistant.
[g] Not determined.

Fig. 5. A, Immunodetection of the fungal, in planta–secreted ECP1 protein with 5 μl of apoplastic fluid (AF) obtained 20 days post inoculation of strain 2679 on the indicated genotypes. Lane 1, MM-Cf0; lane 2, MM-Cf9. **B,** Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 100 μl of AF isolated from leaflets 12 days post inoculation of the strain 2679 on the indicated genotypes. Lane M, molecular weight marker; lane 1, MM-Cf0; lane 2, MM-Cf9.

An example of several linked resistance genes against the same pathogen at one locus is the two copies of the *Cf-2* gene (Dixon et al. 1996), and most of the resistance genes cloned so far have been found to be part of clusters of homologues (Bent 1996). For resistance toward *C. fulvum* in tomato, our data do not suggest that there is an equivalent situation for the *Cf-4* and *Cf-5* gene clusters, as colonization of race 4 and race 5 is similar on MM-Cf0, and on MM-Cf4 and MM-Cf5, respectively. This indicates that homologues different from the functional *Cf-4* and *Cf-5* genes do not govern any resistance to these races, or, alternatively, that race 4 and race 5 have already overcome these additional resistance genes.

There are some natural strains of *C. fulvum* that are able to overcome the functional *Cf-9* resistance gene (Lindhout et al. 1989). Although these strains have been known to exist for more than a decade, plant breeders envisage the *Cf-9* resistance gene as a strong gene. The presence of the additional weak resistance gene(s) probably explains why the strains of *C. fulvum*, reported to be able to overcome the *Cf-9*–mediated resistance, are not causing a threat to tomato crops in practice, as these strains still encounter this weak resistance. Only the most recently isolated natural *Avr9* mutant tested by us was able to overcome the weak resistance.

Different from *Cf-9*–mediated resistance, the weak resistance described here seems not to depend on HR enabling early containment of the fungus (Hammond-Kosack and Jones 1994). Possibly, delayed defense responses allow continuous escape of hyphae, causing a continuous stress to host cells over a large area and leading to unusually high levels of PR protein accumulation. It is most likely that the reported weak resistance is mediated by *Cf-9* homologue(s) present in the introgressed segment, though only transformation of MM-Cf0 tomato with individual homologues would give a definitive proof. Consequently, matching fungal elicitor(s) recognized due to the presence of the additional resistance gene(s) are predicted to exist. Leaf injection bioassay, as previously carried out to identify the AVR9 and AVR4 elicitors, did not trigger a visible response on MM-Cf9 plants. So other assays are required to achieve isolation and characterization of the matching fungal elicitors.

MATERIALS AND METHODS

Strains and media, plants and inoculations.

Culture conditions for the various strains of *C. fulvum* and tomato plants, as well as the inoculation procedure were according to De Wit (1977). The development of symptoms was followed in time and photographed 2 to 3 weeks post inoculation. All inoculations were conducted three times with two plant individuals per fungal strain/plant genotype combination, and we presented pictures of a representative experiment.

Genotypes of plants and fungal strains.

The set of tomato plants tested consisted of the following: the cv. Moneymaker (MM-Cf0), which does not contain any known genes for resistance against *C. fulvum*, and three near-isogenic lines of this cultivar; MM-Cf9, containing an introgression segment of *L. pimpinellifolium* PI126933 carrying the *Cf-9* resistance gene; MM-Cf4, containing an introgression segment of *L. hirsutum* PI370085 carrying the *Cf-4* resistance gene; and MM-Cf5, containing an introgression segment of *L.*

esculentum var. *cerasiforme* PI187002 carrying the *Cf-5* gene (Stevens and Rick 1988). These three genotypes are known to be fully resistant via the introgressed resistance genes, *Cf-9*, *Cf-4*, or *Cf-5*, which are directed toward fungal strains that contain the corresponding avirulence genes *Avr9*, *Avr4*, and *Avr5*, respectively. MM-Cf9/*Cf-9* EMS mutants, M140 and M466 (Hammond-Kosack et al. 1994), were obtained from K. Hammond-Kosack (John Innes Center, Norwich, U.K.). The relevant genotypes of the fungal strains used in this study are the following: race 4 (*Avr4* mutant, *Avr5*⁺, *Avr9*⁺); race 5 (*Avr4*⁺, *Avr5* mutant, *Avr9*⁺); A43ΔAvr9 (*Avr4* mutant, *Avr5*⁺, *Avr9* mutant); B51ΔAvr9 (*Avr4*⁺, *Avr5* mutant, *Avr9* mutant) (Marmeisse et al. 1993). Natural *Avr* mutants NZb and 2679 were obtained from the collections of the Manaaki Whenua Landcare Research, Auckland, New Zealand and the Research Institute for Plant Protection, Wageningen, The Netherlands, respectively. Other natural *Avr9* mutants tested were from the collection of our laboratory.

Light microscopy.

Leaf disks were obtained from leaf material 3 weeks post inoculation. Staining of the intercellular fungal hyphae was as described by Laugé et al. (1997).

Preparation and analysis of AFs.

Six leaflets were randomly sampled from inoculated plants for preparation of AFs according to the method described by De Wit and Spikman (1982). Analysis of total soluble proteins was performed by 15% (wt/vol) SDS-PAGE of 100 µl of freeze-dried AF. For immunodetection of individual PR proteins and the fungal ECP2 protein, the proteins present in 5 µl of AFs were first separated with 15% SDS-PAGE and subsequently transferred to Immobilon-P membrane (Millipore, Etten-Leur, The Netherlands). Antibodies raised against β-1,3-glucanase, chitinase, and the three basic 15-kDa PR proteins have been described previously (Joosten and De Wit 1989; Joosten et al. 1990; Wubben et al. 1994). For immunodetection of ECP1, the proteins present in 5 µl of AF were separated by native high-pH PAGE (pH 8.8), before transfer to Immobilon-P. The antibodies against ECP1 have been described by Joosten and De Wit (1988).

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Chapter 7

General discussion

GENERAL DISCUSSION

Until the late seventies, molecular studies were hardly existing in the field of plant pathology. Most research was focused on physiology, symptomatology and epidemiology of plant diseases (Agrios, 1997). The rationale behind this was that these fields of research were helpful to reduce disease losses.

A growing interest in the genetics of plants and their pathogens appeared concomitantly with the generation of tools in molecular genetics (Agrios, 1997). Presently in research much attention is given to i) the genetics of pathogenicity: understanding why and how are microbes pathogenic, and to ii) the molecular basis of plant resistance: understanding why and how certain plants are resistant, while others are susceptible to disease. By dissecting these two molecular mechanisms, it is anticipated to provide at mid-term some alternative methods to control plant diseases in the field.

Currently, we are still in the period of generating data. However, already considerable molecular and genetic understanding of microbial pathogenicity, plant defense and resistance has been obtained. Concerted research culminated during the past four years with the cloning of the first resistance genes (Bent, 1996). The first concepts for molecular breeding are being tested for their feasibility. In this chapter, the contribution of the results described in the previous chapters to our understanding of the *C. fulvum*-tomato and other plant-pathogen interactions are discussed.

PATHOGENICITY AND VIRULENCE OF FUNGAL PLANT PATHOGENS

In Chapter 3 we studied the putative involvement of the *Ecp*1 and *Ecp*2 genes, encoding ECP1 and ECP2 respectively, in pathogenicity or virulence of *C. fulvum* on tomato (Joosten and De Wit, 1988; Van den Ackerveken *et al.*, 1993). The genes and their products do not share any significant homology with sequences in databases. Both genes were hypothesized to play a role in pathogenicity as they are both strongly induced *in planta*. The importance of each gene was assessed by targeted gene disruption and subsequent comparison of the mutant strain with the wild-type strain from which it was derived. This strategy is believed to allow a reliable assessment of the function of a gene in plant infection, as both tested strains differ only by the gene of interest (Oliver and Osbourn, 1995). It is already known that *Ecp*2 is not a pathogenicity gene as it is dispensable for causing disease on tomato seedlings (Marmeisse *et al.*, 1994). However, in greenhouse and field conditions, *C. fulvum* is known to cause leaf mold on fully developed leaves of plants (Jones and Jones, 1991). Therefore, tomato seedlings are poor hosts for assaying a putative pathogenicity factor of *C. fulvum*. Moreover, the limited size of the material hampered the detection of a quantitative difference. Both genes were found to be virulence genes of the fungus when colonizing leaves of adult tomato plants,

as their absence does not abolish disease but only reduces the parasitic fitness of the fungus. The two definitions that pathogenicity genes/factors are a prerequisite for disease-causing ability and that virulence genes/factors are dispensable for disease-causing ability but have a quantitative effect on parasitic fitness, are rather theoretical (Agrios, 1997). Most research aimed at testing the importance of genes of fungal plant pathogens in pathogenicity by targeted gene disruption, report no or only partial reduction of pathogenicity (Oliver and Osbourn, 1995). Exceptions comprise (i) genes that encode for host-selective toxins (Walton, 1996), (ii) genes whose activity is essential for a pathogen to develop or switch infection structures during the disease cycle (Hamer and Holden, 1997), and (iii) genes involved in detoxification of particular antimicrobial compounds (Osbourn, 1996). Most of the genes encoding enzymes that can degrade plant cell walls or phytoalexins do not seem to be as crucial as thought originally (Oliver and Osbourn, 1995). Apparently, fungal pathogenicity relies more on pyramiding of many virulence factors that are individually dispensable, rather than on just a few pathogenicity factors. This can be foreseen as an advantage for plant-pathogenic fungi, as they can adapt to the plant defense strategies more adequately. The fact that ECP1 and ECP2 are virulence factors for *C. fulvum* is consistent with our reports in Chapters 4 and 5. If the fungus would have had only one extracellular pathogenicity factor, it would probably have already been counter-selected for by a plant exhibiting HR-associated recognition specificity of this factor.

MOLECULAR BASIS OF RACE-SPECIFIC RESISTANCE IN PLANTS

The existence of race-specific resistance has been extensively exploited in commercial plant breeding programs. Most resistance characters in wild relatives of crops have been demonstrated to be monogenic and dominant (Agrios, 1997). Consequently, they represent an easy solution to prevent disease and have been extensively introduced into commercial cultivars. However, often these monogenic resistances were rapidly overcome by new strains of the disease-causing pathogen (Agrios, 1997). Therefore, in the last decade much research was focused on understanding the molecular basis of race-specific resistance in order to develop new and more efficient strategies for durable resistance breeding.

Avirulence genes

More than thirty bacterial avirulence genes, and eight fungal avirulence genes have been cloned and characterized in the past ten years. As reviewed by Leach and White (1996) and Alfano and Collmer (1996) for bacteria and described in Chapter 2 for fungi, they all are highly diverse in sequence, in regulation, and in the products they encode. Clearly, there is no real profile of an "avirulence gene". The only limitation to the nature

of an avirulence gene of a given pathogen seems to rely on the way the pathogen infects plants. Plant pathogenic bacteria that use a type III secretion system, produce cytoplasm- or nucleus-targeted proteins that act as avirulence factors, while fungi and oomycetes that often have an intercellular biotrophic stage produce predominantly secreted extracellular proteins that act as avirulence factors.

The *C. fulvum*-tomato interaction has been used as model that complies with the gene-for-gene system (De Wit, 1992). The fungus remains confined to the apoplastic space during the whole disease cycle (De Wit, 1977). Thus, race-specific resistance of tomato cultivars had been hypothesized to operate through recognition of molecules that are secreted by the pathogen during infection. This working hypothesis could be confirmed by the isolation and analysis of the two extracellular race-specific elicitors, AVR4 and AVR9 (Scholtens-Toma and De Wit, 1988; Joosten *et al.*, 1994). The cloning of the two encoding genes, *Avr4* and *Avr9*, and their genetic transformation into, respectively, Avr4⁻ and Avr9⁻ strains of the fungus, demonstrated unequivocally that they represent the two avirulence genes matching the corresponding tomato resistance genes, *Cf-4* and *Cf-9*, respectively (Van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). Other extracellular proteins secreted by the fungus during infection had already been identified, ECP1 and ECP2 (Joosten and De Wit, 1988; Wubben *et al.*, 1994), or were isolated as described in this thesis. In Chapters 4 and 5, we tested several ECPs for their ability to act as specific elicitors on yet unidentified tomato cultivars and/or genotypes of *Lycopersicon* species. For each of these ECPs tested we could find at least one individual exhibiting HR upon exposure to the protein, in the same manner as has been described for Cf4 and Cf9 genotypes responding to AVR4 and AVR9, respectively. Furthermore, using a fungal mutant strain in which the *Ecp2* gene has been deleted, we demonstrated that ECP2 is the only factor responsible for the resistance exhibited by the tomato cultivar that specifically recognizes ECP2. In other words, *Ecp2* is a genuine avirulence gene of *C. fulvum*. Similar experiments have to be carried out to show whether the genes encoding the other ECPs are also avirulence factors. Thus, we could confirm that HR-associated recognition of proteins secreted by *C. fulvum* confers resistance to tomato.

We limited our search to specific proteinaceous elicitors that are able to induce HR in plants. Studies on other plant-pathogen interactions following the gene-for-gene system have already demonstrated that other types of avirulence factors exist, such as HR-inducing syringolides (Keen *et al.*, 1990), while it has also been reported that there are proteinaceous elicitors that trigger defense responses other than HR (Hahn *et al.*, 1993; Rohe *et al.*, 1995). Chapter 6 describes the occurrence of additional resistances present on the *Cf-9* introgression segment that contains a cluster of *Cf-9* homologues. It has been demonstrated that certain *Hcr9-9* genes (<u>H</u>omologue of <u>C</u>ladosporium fulvum <u>R</u>esistance gene *Cf-<u>9</u>* present at the *Cf-<u>9</u>* locus) confer the identified additional resistances (Parniske *et al.*, 1997) and give a high level of accumulation of PR proteins. So far, we failed to isolate a HR-inducing avirulence factor corresponding to the *Hcr9-9* genes. These results

point out that elicitors inducing non HR-based resistance might occur in the *C. fulvum*-tomato interaction.

Resistance genes

Considerable research in the past decade has focused on cloning of plant resistance genes. *Pto,* that confers resistance to tomato against *Pseudomonas syringae* pv. *tomato*, was the first gene-for-gene-based resistance gene of which the sequence was obtained by Martin *et al.* (1993). Since then, about twenty resistance genes against pathogens as diverse as viruses, bacteria, fungi, nematodes and aphids, have been cloned from tomato, Arabidopsis, tobacco, flax, sugarbeet and rice (Bent, 1996; Hammond-Kosack and Jones, 1997; De Wit, 1997). They all share extensive structural similarities and contain similar building blocks: leucine-rich-repeats (LRR), leucine zipper domains, nucleotide-binding sites and kinase domains. They are all members of gene families organized in only a few clusters containing several homologues. Four tomato resistance genes against *C. fulvum*, *Cf-2, Cf-4, Cf-5,* and *Cf-9,* have been cloned (Jones *et al.*, 1994; Dixon *et al.*, 1996; Thomas *et al.*, 1997; Dixon *et al.*, 1998). They all encode LRR-proteins predicted to be extracellular and membrane-anchored. Clusters of *Cf* homologues are present on chromosomes 1 and 6 of tomato.

The organization of resistance genes is reminiscent of that of the highly flexible major histocompatibility complex of mammals (Parniske *et al.*, 1997; Steinmetz *et al.*, 1982). Nucleotide sequence comparisons of resistance gene homologues indicate that extensive sequence polymorphism is achieved by gene duplication, gene conversion and unequal crossing-overs. This extreme plasticity of the nucleotide sequences is hypothesized to generate highly variable recognition specificities. Recently cloned plant genes such as *PRK1* of Petunia, *ERECTA1*, *BRI1* and *CLAVATA1* of Arabidopsis, *CRINKLY4* of maize and *SERK* of carrot are receptor-like kinases that are highly homologous to resistance genes but are involved in regulation of plant development (Becraft, 1998). Remarkably, some of these genes are hypothesized to have proteinaceous ligands. Taken together, the current data suggest that these genes have a common origin and enable plants to perceive both external and internal signals. The system for recognizing and decoding internal signals might have evolved into a surveillance system, responsible for perception of external non-self molecules such as fungal elicitors. While internal signals could regulate signal transduction pathways leading to the activation of developmental genes responsible for switches in plant development, external signals could induce specific types of HR (activation of defense-related genes and/or activation of programmed cell death) which would efficiently protect plants against pathogenic intruders.

So far, no *Cf* homologue has been demonstrated to be involved in development of tomato. However, as discussed above, the *Hcr9-9* genes giving additional resistances against *C. fulvum*, might not recognize proteinaceous fungal elicitors but rather fungal secondary metabolites. Noteworthy, secondary plant metabolites, such as the

brassinosteroids, are suggested to be directly bound by the *BRI1* gene product (Li and Chory, 1997). Moreover, the *CLAVATA3* gene which, on the basis of genetic studies, is thought to encode the putative ligand of the *CLAVATA1* gene product, has been cloned recently (Fletcher *et al.*, 1998). It encodes a small protein reminiscent of the AVR and ECP proteins which are the putative ligands of the Cf proteins, alone or as part of a complex. Overall, it appears that the perception of internal signals during development of plants and the perception of external signals during pathogen attack might function in a very similar way.

The polymorphic sequences present in clusters of resistance gene homologues have been examined extensively at the *Cf-4/Cf-9* locus in tomato (Parniske *et al.*, 1997) and at the *Xa21* locus in rice (Song *et al.*, 1997). Both clusters have undergone extensive sequence rearrangement, probably as a result of duplication, homologous recombination, unequal crossing-over and gene conversion in intergenic and intragenic sequences. Clear selection for sequence diversification has occurred in the N-terminal part of the LRR-domain, which is predicted to interact with the corresponding avirulence ligands. Results obtained in Chapters 4 and 5 support this model, as we could always find tomato or *Lycopersicon* genotypes that recognize a particular ECP as a specific elicitor. Not surprisingly, mapping of the *Cf-ECP2* gene revealed that it is closely linked to the *Cf-4/Cf-9* cluster on the short arm of chromosome 1 (Haanstra *et al.*, in preparation). Thus, the reported diversity of resistance gene homologues and our findings on the *C. fulvum*-tomato interaction both indicate the versatility of recognition specificities of LRR proteins encoded by the *Cf* genes and their homologues.

CONSEQUENCES FOR THE CURRENT VIEW ON GENE-FOR-GENE SYSTEMS

The existence of a highly versatile surveillance system in plants for non-self molecules would slightly modify the current view on gene-for-gene systems. According to the hypothesis discussed above, the plant surveillance system would rather generate random recognition specificities than targeted recognition specificities aimed at actual pathogens. Similarly to the distribution of antigen recognition capacities among B cell lines of the mammalian immune system (Tonegawa, 1983; Manser *et al.*, 1984), plant recognition specificities would be scattered in populations of plants in which each individual would encode a definite range of "antigen"-recognizing receptors. As discussed in Chapter 5, with one single plant and one single strain of a pathogen, resistance will take place when the pathogen produces a molecule that can be recognized by the plant. If no recognition occurs, the recognition specificities of the plant would remain unrevealed and the defense system would not be activated. In a few studies on population genetics, a population of plants has been assayed for its resistance spectrum against a collection of pathogenic strains either sympatric (same geographical origin) or allopatric (different geographical

origin) (Burdon, 1987; Lawrence and Burdon, 1989; Cattan-Toupance, 1997; Cattan-Toupance *et al.*, 1998). While resistance tests against sympatric strains revealed the existence of many independent resistances, surprisingly, resistance tests against allopatric strains showed nearly as many, albeit distinct resistances. Thus, numerous gene-for-gene pairs do occur within one particular plant-pathogen relationship and are not confined to plants and pathogens that share the same geographical origin.

The diversity of recognition specificities seems to depend on the number of putative elicitor molecules produced by the pathogen during infection. Thus, the number of potential avirulence genes would be determined by the "antigenicity" of its products. At a certain point, saturation would occur in the system with new resistance genes matching known avirulence genes, and vice versa. Accordingly, the classical examples where numerous resistance/avirulence gene pairs within the same plant-pathogen interaction occur: flax-*Melampsora lini*, tomato-*C. fulvum*, wheat-*Puccinia graminis*, potato-*Phytophthora infestans*, lettuce-*Bremia lactucae*, refer to fungal or oomycete pathogens rather than to bacteria and viruses. Also screening of germplasms often results in a higher number of accessions with a functional resistance character against bacteria than against viruses, or against fungi than against bacteria (Fucillo *et al.*, 1997). Therefore, in most cases there is a positive correlation between the number of functional resistances found in the host plant and the degree of complexicity of the genome of the pathogen. Some pathogens that exert a very strong selection pressure on their host might bias the data, as plants with a resistance against these pathogens would likely spread in the population due to natural selection. Our data described in Chapter 5, where we found plants responding with HR to any of the ECP proteins, support this hypothesis. Furthermore, we found several genotypes of apparently different origin that respond to the same protein (Laugé *et al.*, unpublished; Joosten *et al.*, unpublished). This might be an indication for saturation of the system. As long as the gene(s) responsible for the recognition of the same protein are not isolated and characterized, we can only speculate about the independent occurrence of these genes. However, the fact that HR-associated recognition of certain AVR/ECP proteins occurs in accessions of *L. pimpinellifolium* that originate from distal locations within the genetic centre of this species, gives some support to this hypothesis (Laugé *et al.*, unpublished).

The model also predicts that recognition specificities in plants are generated randomly and are not limited to molecules originating from their "own" pathogenic microorganisms. Our results presented in Chapter 5, where we described that accessions of *Nicotiana* species recognize ECP2 as a specific elicitor of HR, support this model, although we have not investigated the possibility that ECP2 or a functional ECP2-like molecule occurs in pathogens of tobacco. If not, this recognition should not be selected for, and could be soon lost from the *N. paniculata* population. It appears now particularly interesting to test whether we can detect HR-associated recognition in the tomato germplasm of extracellular AVR/ECP-like proteins originating from non-pathogenic

microorganisms, insects or even mammals. When pushed to the extreme, plant individuals will sometimes also exhibit recognition specificities against some of their own proteins. Obviously, these plants would display a type of "auto-immune" response in the first stages of their life, and they would be eliminated from the population. It could be that some of the lesion-mimic mutants reported in literature for corn, Arabidopsis and tomato (Dangl *et al.*, 1996) may represent individuals that respond to their "own" proteins at certain stages of plant development.

BIOTECHNOLOGICAL PERSPECTIVES

Many efforts currently aim at the exploitation of gene-for-gene pairs in genetically-engineered resistance. Several concepts, such as the two-component system (De Wit, 1992), the genetically engineered acquired resistance (Hammond-Kosack, 1998) have shown to be promising and are being optimized for their ability to confer broad-range disease protection. An alternative approach that does not require genetic transformation is to identify resistance genes that operate through recognition of factors of a pathogen that are important or required for pathogenicity and/or virulence. Confronted with such a resistance gene, a pathogen could probably not readily adapt without compromising its parasitic fitness. Our data, presented in Chapter 4, illustrate this concept for *Cf-ECP2* which is targeted against the ECP2 protein, previously shown to be an important virulence factor for *C. fulvum*. Breeding for efficient resistance against economically important pathogens such as mildews, rusts or oomycetes would benefit of such a strategy, provided that pathogenicity/virulence targets for this type of resistance can be identified in the pathogen. Obviously, the limitation of this approach is that it cannot be employed for pathogens that are not inhibited by HR, as is the case for necrotrophic pathogens like *Botrytis cinerea*.

In another approach, the accumulating knowledge about resistance genes is employed to create artificial "mosaic/hybrid" resistance genes with a desired recognition specificity. However, it might be that the perfect gene already exists in wild accessions yet to be identified. Once more screening vectors are available (such as PVX, which can be used for solanaceous species), breeders and plant pathologists will have tools to screen a broad spectrum of plants for potentially more durable types of resistance.

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SUMMARY

The interaction between the biotrophic fungus *Cladosporium fulvum* (Cooke) and its only host, tomato (*Lycopersicon esculentum* Miller) complies with the gene-for-gene model. Early genetic studies have shown that many different avirulence (*Avr*) and resistance (*R*) gene pairs exist between strains of the fungus and genotypes of the plant, respectively. Previous studies in our laboratory, as summarized in Chapter 1, led to the isolation and characterization of several proteins of fungal and plant origin that occur in the apoplast during infection. The search for proteinaceous fungal elicitors that could act as avirulence factors on resistant tomato genotypes, led to the isolation of two proteins, AVR4 and AVR9. The development of molecular tools to manipulate *C. fulvum* provided means to confirm that the two encoding genes, *Avr4* and *Avr9*, are the avirulence genes that match the tomato resistance genes *Cf-4* and *Cf-9*, respectively. The genes encoding two additional fungal extracellular proteins (ECPs), *Ecp1* and *Ecp2*, have also been cloned. Based on their specific induction *in planta*, both genes were proposed to be putative pathogenicity genes.

The object of the research presented in this thesis was to obtain a better understanding of the function of the ECPs in pathogenicity and virulence of *C. fulvum* on tomato and of their potential role as specific elicitors on particular tomato genotypes, which would possibly fit into additional *Avr/R* gene-for-gene pairs. Accordingly, most of the work presented in this thesis involves studies on the biological function of the ECP1 and ECP2 proteins and additionally characterized ECPs of *C. fulvum*.

In Chapter 2 a review on our current knowledge on fungal avirulence genes is presented. Although only a few fungal *Avr* genes have been cloned so far, they encode very different proteins and are differently regulated. Their involvement in pathogenicity or virulence, the type of responses that their products trigger on plants carrying the matching resistance genes, and their potential application in molecular resistance breeding are discussed.

Pathogenicity assays with transgenic strains of *C. fulvum* in which the *Ecp1* and/or *Ecp2* genes were deleted are presented in Chapter 3. Both genes are virulence factors, as their encoded products, ECP1 and ECP2, are required for full virulence. Nevertheless, when absent, the fungus is still able to cause disease on tomato. The studies also indicate that both genes might function as suppressors of plant defense responses. It is envisaged that pathogenicity of *C. fulvum* on tomato results from synergism between multiple virulence factors. That is, while each single virulence factor is partly involved in causing disease on tomato, the presence of a complete set of virulence factors ensures maximal parasitic ability.

Both ECP1 and ECP2 proteins are secreted, have a low molecular weight and are cysteine-rich proteins. They are secreted early and abundantly in the intercellular space, in

a similar manner as observed for the two race-specific elicitors, AVR4 and AVR9. In Chapter 4 a search for plant genotypes that could recognize these proteins as specific elicitors of HR is described. One genotype of tomato, which had been preselected for resistance to *C. fulvum*, showed specific HR upon exposure to ECP2. Significantly, resistance of this genotype was shown to rely solely on ECP2 recognition. The resistance character is conferred by a single dominant gene, designated *Cf-ECP2*. Analogous to the *Avr4/Cf-4* and *Avr9/Cf-9* gene pairs, *Ecp2/Cf-ECP2* represents a genuine gene-for-gene pair within the *C. fulvum*-tomato interaction. Since the *Cf-ECP2* gene operates through recognition of a crucial virulence factor, it may, therefore, provide durable protection against *C. fulvum* in tomato crops. Thus, the same protein may have a role in virulence on all tomato plants and a role in avirulence on only particular tomato genotypes.

In Chapter 5, we subsequently initiated an extensive screen for plants among breeding lines and wild accessions of *Lycopersicon* species that could respond specifically with HR to any of the five interaction-specific fungal ECPs that we had isolated. Indeed, for each of the five ECPs that were tested, we found at least one individual that responded with HR. This observation indicates that recognition specificities are scattered among populations of plants, suggesting that recognition of proteins of *C. fulvum* is a generic property of the *Lycopersicon* genus. To investigate whether this is also true for a non-host species of *C. fulvum*, wild accessions of *Nicotiana* spp. were screened for similar specific HR-associated recognition of proteins of *C. fulvum*. We identified a few *N. paniculata* plants that exhibited a specific HR towards ECP2. This suggests that generic recognition specificities of proteinaceous elicitors of a fungal pathogen exist in solanaceous plants outside its host range. Consequently, the plant system for recognition of foreign proteins seems to generate new specificities at random, rather than specificities against classes of proteins produced by the pathogens of a given host plant.

Recently, the cloning of four tomato *Cf* genes, among which are the *Cf-4* and the *Cf-9* genes that match the avirulence genes *Avr4* and *Avr9* respectively, was reported. They all belong to the same gene family, which is predicted to encode membrane-anchored leucine-rich-repeat (LRR) proteins. As such, they might participate in the perception of the different fungal avirulence factors. The *Cf* genes are members of clusters containing several homologues. In Chapter 6 we tested whether some of the *Cf-9* homologues, referred to as *Hcr9-9* genes (Homologue of *Cladosporium fulvum* Resistance gene *Cf-9* present at the *Cf-9* locus), could confer additional resistance against *C. fulvum*. Inoculation of fungal strains in which the *Avr9* gene was deleted onto Cf9 tomato genotypes, showed that additional partial resistance(s) are present on the *L. pimpinellifolium* genomic segment introgressed into *L. esculentum*. These additional resistance(s) are independent of the *Avr9/Cf-9* gene pair and are most probably due to one or more of the *Hcr9-9* genes. However, we could not identify any specific proteinaceous elicitor activity in apoplastic fluids of compatible *C. fulvum*/tomato interactions that could match the identified additional resistance(s). The suggestion that *Hcr9-*

9 genes are responsible for the additional resistance(s) has been proven in an independent study in another research group. Therefore, the *Lycopersicon* genus is not only rich in recognition specificities for secreted *C. fulvum* proteins (AVRs and ECPs), also a single plant genotype appears to contain multiple recognition capacities towards a single fungal strain.

The data obtained in this research are discussed in Chapter 7. They indicate that HR-associated recognition of extracellular, low-molecular weight, cysteine-rich proteins of *C. fulvum*, some of which are involved in pathogenicity, is a general phenomenon in the *Lycopersicon* genus. The finding that some of these proteins, that act as avirulence factors, are also important virulence factors of the fungus might help to develop a strategy for identification of putative durable resistance genes, as proposed for the *Cf-ECP2* gene. The demonstration of partial resistance(s) conferred by the *Cf-9* homologues, without detection of a HR-phenotype, could suggest that recognition capacities of these genes are not limited to proteins, or that gene-for-gene responses other than HR occur in the *C. fulvum*-tomato interaction. In addition, our data indicate that HR-recognition of *C. fulvum* proteins is not confined to its host genus, *Lycopersicon*, but also occurs in the non-host genus *Nicotiana*.

In conclusion, it appears that the diversity of the HR-associated recognition of proteins by a variety of *Cf* genes in a population of tomato relatives, generates plants with a genuine surveillance system against invading pathogens. The importance of the recognized proteins in pathogenicity or virulence of the pathogen is likely to be positively correlated with the durability of the matching resistance gene. The wide occurrence of gene-for-gene systems in crop plants is most probably a result of plant breeding activities by humans, involving transfer and dissection of gene clusters that originate from wild species.

SAMENVATTING

De interactie tussen de biotrofe schimmel *Cladosporium fulvum* (Cooke) en zijn enige gastheer, tomaat (*Lycopersicon esculentum* Miller), past in het model van een gen-om-gen relatie. Uit genetische studies is gebleken dat verschillende isolaten van de schimmel diverse avirulentie (*Avr*) genen bezitten, terwijl in verschillende genotypen van tomaat diverse resistentie (*R*) genen voorkomen. Deze *Avr* en *R* genen kunnen bovendien in allerlei combinaties voorkomen.

Zoals samengevat in hoofdstuk 1, heeft voorafgaand onderzoek in het Laboratorium voor Fytopathologie geleid tot de isolatie en karakterisering van diverse schimmel- en planteiwitten, die aanwezig zijn in de apoplast van gekoloniseerde bladeren. Apoplastische schimmeleiwitten kunnen door bepaalde lijnen van tomaat als avirulentiefactoren herkend worden en resistentie induceren. Twee van deze elicitoren, AVR4 en AVR9, werden reeds einige jaren geïsoleerd. Door middel van genetische modificatie van *C. fulvum*, kon worden bevestigd dat de coderende genen, *Avr4* en *Avr9*, de avirulentiegenen zijn welke corresponderen met respectievelijk de resistentiegenen *Cf-4* en *Cf-9* van tomaat. De genen coderend voor twee andere extracellulaire schimmeleiwitten (ECPs), *Ecp1* en *Ecp2*, waren ook reeds gekloneerd. De waarneming dat de expressie van deze genen in de schimmel sterk wordt geïnduceerd tijdens de kolonisatie van de plant, suggereert dat deze genen pathogeniteitsgenen zijn.

Het in dit proefschrift beschreven onderzoek had als doel meer inzicht te krijgen in de rol van de ECPs in de pathogeniteit van *C. fulvum* op tomaat. Bovendien werd hun rol als mogelijke specifieke elicitoren op diverse genotypen van tomaat onderzocht, waardoor additionele *Avr/R* genenparen zouden kunnen worden geïdentificeerd. Het merendeel van het in dit proefschrift beschreven werk betreft studies aan de biologische functie van de ECP1 en ECP2 eiwitten. Daarnaast wordt de isolatie en karakterisering van andere ECPs van *C. fulvum* beschreven.

Hoofdstuk 2 geeft een overzicht van onze huidige kennis op het gebied van avirulentiegenen van schimmels. De *Avr* genen die tot nu toe zijn gekloneerd coderen voor zeer uiteenlopende eiwitten en hun expressie wordt zeer verschillend gereguleerd. Het hoofdstuk behandelt de rol van deze eiwitten in de virulentie van de schimmel en de inductie van resistentie in planten die het corresponderende resistentiegen bezitten. Bovendien wordt het type responsen dat wordt geïnduceerd in deze planten beschreven en wordt een mogelijke toepassing van avirulentie- en resistentiegenen in moleculaire resistentieveredeling behandeld.

Pathogeniteitstesten met transgene isolaten van *C. fulvum* waaruit het *Ecp1* en/of het *Ecp2* gen is verwijderd, worden beschreven in hoofdstuk 3. Het blijkt dat de gecodeerde eiwitten, ECP1 en ECP2, noodzakelijk zijn voor volledige pathogeniteit van de schimmel op tomaat en dus kunnen worden beschouwd als virulentiefactoren. Als de genen afwezig zijn, is de schimmel echter nog steeds in staat zwakke ziektesymptomen te veroorzaken. Verdere studies

wijzen erop dat de ECP1 en ECP2 eiwitten mogelijk functioneren als suppressors van de afweerresponsen van de plant. Het lijkt er op dat de pathogeniteit van *C. fulvum* op tomaat berust op een samenspel van de verschillende virulentiefactoren; elke afzonderlijke virulentiefactor is betrokken bij het ziekteproces, terwijl de aanwezigheid van een complete set van virulentiefactoren de schimmel een maximale pathogeniteit verschaft.

De ECP1 en ECP2 eiwitten hebben beide een laag moleculgewicht, zijn cysteïne rijk en worden door *C. fulvum* al tijdens de penetratie van het bladweefsel in relatief grote hoeveelheden uitgescheiden in de apoplast. Deze eigenschappen zijn zeer vergelijkbaar met die van de fysio-specifieke elicitoren AVR4 en AVR9. In hoofdstuk 4 wordt de identificatie van tomatengenotypen beschreven die het ECP1 en/of het ECP2 eiwit herkennen en vervolgens een overgevoeligheidsreaktie geven. Een genotype, welke reeds in de zeventiger jaren was geselecteerd op resistentie tegen *C. fulvum*, gaf een specifieke overgevoeligheidsreactie (HR) na herkenning van ECP2. Het ECP2 eiwit bleek de enige factor van de schimmel te zijn die werd herkend door deze plant. Bovendien bleek deze herkenning het gevolg te zijn van de aanwezigheid van een enkel dominant gen, *Cf-ECP2* genaamd. Op deze manier representeren *Ecp2* en *Cf-ECP2* een nieuw gen-om-gen paar binnen de interactie tussen *C. fulvum* en tomaat, dat vergelijkbaar is met de eerder geïdentificeerde genenparen *Avr4/Cf-4* en *Avr9/Cf-9*. Daar het *Cf-ECP2* resistentiegen functioneert via herkenning van een essentiële virulentiefactor van de schimmel, kan het gen mogelijk zorgdragen voor een duurzame bescherming van tomaat tegen *C. fulvum*. Hiermee is aangetoond dat hetzelfde eiwit zowel een rol kan spelen in virulentie op de waardplant, als in avirulentie op bepaalde genotypes van de plant.

In het onderzoek beschreven in hoofdstuk 5 is vervolgens een uitgebreide screening opgezet ter identificatie van individuen uit veredelingslijnen en accessies van wilde soorten van *Lycopersicon*, die specifiek met een HR reageren op een of meerdere van de in totaal vijf geïsoleerde ECPs. Er werd, voor elk van de vijf ECPs, tenminste één individu gevonden dat reageerde met een overgevoeligheidsreactie. Deze waarneming wijst erop dat het vermogen om de ECPs specifiek te herkennen, verspreid is over de gehele populatie van planten en dat het herkennen van uitgescheiden eiwitten van *C. fulvum* een algemene, intrinsieke eigenschap van het geslacht *Lycopersicon* is. Om te toetsen of dit bovendien voor niet-waardplanten geldt, werden accessies van diverse wilde *Nicotiana* soorten getest op het geven van een vergelijkbare HR na herkenning van de diverse extracellulaire eiwitten van *C. fulvum*. Er werden op deze manier enkele *N. paniculata* planten geïdentificeerd die een specifieke HR gaven tegen ECP2. Deze waarneming impliceert dat, zelfs buiten de waardplantreeks van een pathogeen, in de familie van de *Solanaceae* elicitoren van *C. fulvum* herkend kunnen worden. Het systeem waarmee een plantenpopulatie vreemde 'antigene' eiwitten herkent, lijkt diverse herkenningspecificiteiten reeds latent te bezitten, in plaats van deze nieuw te ontwikkelen na blootstelling aan een pathogeen.

Recentelijk is de klonering en karakterisering van vier *Cf* genen van tomaat, waaronder de *Cf-4* en *Cf-9* genen die corresponderen met respectievelijk de avirulentiegenen *Avr4* en *Avr9*, gepubliceerd. Ze behoren allemaal tot de familie van membraan-gebonden eiwitten waarin geconserveerde delen voorkomen die rijk zijn aan leucine residuen. Mogelijk maken deze eiwitten deel uit van een complex dat zorgt voor de perceptie van de verschillende avirulentiefactoren van de schimmel. De *Cf* genen zijn aanwezig in clusters waarin diverse homologe genen aanwezig zijn. In hoofdstuk 6 is gekeken of de diverse *Cf-9* homologen, *Hcr9-9* genen genoemd naar homologen van resistentiegen *Cf-9* tegen *C. fulvum*, aanwezig op het *Cf-9* locus, ook resistentie kunnen geven tegen bepaalde isolaten van *C. fulvum*. Inoculatie van tomatenlijnen die het genomische segment bezitten waarop zich het *Hcr9-9* locus bevindt, met schimmelisolaten waaruit het *Avr9* gen verwijderd is, wees er op dat op dit segment inderdaad één of meerdere, zij het partiële, resistentiegenen aanwezig zijn. Deze resistentie is onafhankelijk van het *Avr9/Cf-9* genenpaar en is waarschijnlijk het gevolg van de aanwezigheid van een of meer van de *Hcr9-9* homologen. Er kon echter geen elicitoractiviteit worden geïdentificeerd in apoplastische vloeistof afkomstig van compatibele tomaat-*C. fulvum* interacties, welke correleerde met de additionele resistentie. Dat de *Hcr9-9* genen inderdaad verantwoordelijk zijn voor de waargenomen partiële resistentie, is aangetoond door een andere onderzoeksgroep. Het geslacht *Lycopersicon* is zodoende niet alleen rijk aan herkenningscapaciteit voor gesecreteerde eiwitten van *C. fulvum* (AVRs en ECPs), maar bezit ook binnen één bepaald genotype meerdere herkenningsmogelijkheden van één specifiek schimmelisolaat.

De resultaten die in dit onderzoek werden verkregen, worden verder bediscussieerd in hoofdstuk 7. De waarnemingen wijzen er op dat specifieke herkenning van extracellulaire, cysteine-rijke eiwitten van *C. fulvum* met een laag molecuulgewicht, een algemeen voorkomend fenomeen is binnen het geslacht *Lycopersicon*. Het feit dat een aantal van deze eiwitten belangrijke factoren voor pathogeniteit, virulentie en avirulentie van de schimmel zijn, verschaft mogelijkheden voor de isolatie van potentieel duurzame resistentiegenen, zoals werd voorgesteld voor het *Cf-ECP2* gen. De waarneming dat partiële resistentie, afkomstig van de *Cf-9* homologen, niet leidt tot HR, kan erop wijzen dat in dit geval herkenning leidt tot een andere afweerreactie dan HR. De waarnemingen suggereren bovendien dat zulke herkenningscapaciteiten zich niet beperken tot waardplanten.

Resumerend lijkt het er op dat de variatie in HR-gerelateerde herkenning van eiwitten door een grote verscheidenheid aan *Cf* genen binnen een populatie van tomaat, planten in staat stelt zich te verdedigen tegen potentiële pathogenen. Het belang van deze eiwitten in de pathogeniteit of virulentie is waarschijnlijk positief gecorreleerd met de duurzaamheid van het corresponderende resistentiegen. Het veelvuldig voorkomen van gen-om-gen systemen in cultuurgewassen is het resultaat van resistentieveredeling, waarbij overdracht en opsplitsing van genenklusters, afkomstig van wilde soorten, regelmatig plaatsvindt.

During my first six months, I got initiated to the *C. fulvum*/tomato system in the Laboratory of Genetics. I thank Henk van den Broek, Theo Goosen and Guido van den Ackerveken for the guidance of my first steps in Wageningen. I thank Rik Lubbers for horticultural assistance, Tony van Kampen for the sequencing work, Ali, Elly, Ria, Boukje and Willem for the administrative part, and the members of Duotone for the photographic work.

Finally, support outside the work was also a key point. I am very grateful to Valérie, Franck, Laurence, Isabelle and Laurent, as well as my family and friends from my former french scientific environment. They have encouraged me and contributed to my progresses in my Dutch experience.

LIST OF PUBLICATIONS

Most of the results presented in this thesis have been published or will be published in the near future. The various chapters of this thesis are based on most of the following publications:

Honée G., Van den Ackerveken G.F.J.M., Van den Broek H.W.J., Cozijnsen T.J., Joosten M.H.A.J., Laugé R., Kooman-Gersmann M., Vervoort J., Vogelsang R., Vossen P., Wubben J.P., and De Wit P.J.G.M. (1994) Molecular characterization of the interaction between the fungal pathogen *Cladosporium fulvum* and tomato. In *Advances in Molecular Genetics of Plant-Microbe Interactions* (Daniels M.J., Downie J.A., and Osbourn A.E., eds.) Kluwer academic Publishers, Dordrecht, pp. 199-206.

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Laugé, R., Joosten, M.H.A.J., and De Wit, P.J.G.M. Specific cell death-associated recognition of secreted proteins from a fungal pathogen occurs in host and non-host plants. *in preparation.*

Curriculum vitae

Richard Laugé was born on august 7th 1969 in Soissons, France. In 1986 he obtained his baccalaureate in Sciences from the Lycée Henri Martin, Saint-Quentin, France. After three years of classes preparatoires, he entered the Institut National Agronomique Paris-Grignon in 1989. In 1991/1992, he did a third year specialization in microbiology at Université Laval, Québec City, Québec, Canada. During his stay in Canada, he worked as a student and a technical assistant on the genetics of opine metabolism in *Rhizobium meliloti* and the genetics of virulence in *Agrobacterium tumefaciens*. The following year, in 1992/1993, he did his Diplome d'Etudes Approfondies (DEA) at Université Paris-Sud/XI, Orsay, France in molecular plant pathology. His practical period took place in the Institut de Génétique Microbienne, Université Paris-Sud/XI where he worked on the molecular biology of fungal bacterial-type transposable elements of *Fusarium oxysporum*. In January 1994, he started his PhD study supported by the European Community-Human Capital and Mobility (program ERBCHRXCT930244). The first six months he was in the Laboratory of Genetics (Dr. H.W.J. Van den Broek), and the last four years in the Laboratory of Phytopathology (Prof. Dr. P.J.G.M. De Wit, Dr. M.H.A.J. Joosten), both of the Wageningen Agricultural University, where he worked on the project described in this thesis. Since May 1998, he works as a senior researcher in the Laboratory of Phytopathology.