The role of AVR4 and AVR4E proteins in virulence and avirulence of the tomato pathogen *Cladosporium fulvum*

Molecular aspects of disease susceptibility and resistance

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Chapter 1

General introduction and outline of the thesis

The general introduction will be published by Nienke Westerink, Matthieu H. A. J. Joosten and Pierre J. G. M. de Wit (2002) in “Fungal Disease Resistance in Plant-Biochemistry, Molecular Biology and Genetic Engineering”, edited by Z.K. Punja.

General introduction and outline of the thesis

SUMMARY

A large variety of fungal avirulence (*Avr*) genes has been identified to encode proteins that trigger defense responses in plants carrying the complementary resistance (*R*) gene. It appeared that several pathogens circumvent this *R* gene-mediated resistance by mutation of the active *Avr* gene into an inactive allele. Loss of the avirulence determinant, however, might be associated with a reduced virulence of the pathogen, as some *Avr* genes contribute to virulence either by suppressing (basal) defense responses or by interacting with host-derived virulence targets. Although the primary function of *Avr* genes involves virulence rather than avirulence, the actual contribution to virulence has not been substantiated for the majority of fungal *Avr* genes. Detailed analysis of gene-for-gene pairs has provided further insight as to how and where AVR proteins are recognized by resistant plant genotypes. It appeared that a direct interaction between AVR and R proteins is an exception rather than a rule. Several models have been proposed supporting an indirect interaction between AVR and R proteins and the involvement of at least a third component in the recognition complex.

INTRODUCTION

Pathogenic fungi use diverse strategies to ingress their host plants. Some pathogens enter plants through wounds or natural openings, while others use specialized structures, such as appressoria, to penetrate intact plant surfaces or enter the host using cuticle- and cell wall-degrading enzymes. Most fungal pathogens colonize all plant organs, like leaves, stems, and roots, either by growing between the cells as intercellular mycelium or by penetrating cells and subsequently growing as intracellular mycelium. Some fungi kill their host and feed on dead tissue (necrotrophs), while others colonize the living host (biotrophs) or even require living tissue to complete their life cycle (obligates). During the biotrophic phase, signal and nutrient exchange between pathogen and host is often mediated by specialized infection structures, such as haustoria.

Most plants are resistant toward the majority of pathogenic fungi. A common and effective durable type of resistance is non-host resistance that prevents plants from becoming infected by potential pathogens. Non-host resistance often involves a protection provided by physical barriers or by early signaling events and highly localized responses

within the cell wall (Heath, 2000). Host resistance, however, is usually restricted to a particular pathogen species and is commonly expressed against specific pathogen genotypes. In this case, the plant specifically recognizes the invading pathogen and active defense responses are induced that lead to resistance. Elicitation of defense responses is mediated by the perception of pathogen signal molecules encoded by avirulence (*Avr*) genes, only when the matching plant resistance (*R*) gene is present, which results in an incompatible interaction between host (resistant) and pathogen (avirulent). If the *R* and/or *Avr* gene are absent or non-functional, the interaction between host (susceptible) and pathogen (virulent) is compatible. Opposed to the basal defense responses that often partially inhibit pathogens during colonization of the host plant, *R* gene-mediated resistance involves a rapid and effective defense mechanism that is often associated with a localized death of plant cells, called the hypersensitive response (HR).

Opposed to race-specific elicitors encoded by *Avr* genes, race-nonspecific (or general) elicitors stimulate defense responses in all genotypes of at least one plant species. These general elicitors are not direct products of *Avr* genes, but rather structural fungal cell wall components (like chitin- or glucan oligosaccharides) released by plant hydrolytic enzymes (Nürnberger, 1999). In this chapter, we will focus on fungal *Avr* gene products (race-specific elicitors) that confer species- or genotype-specific resistance. The function of *Avr* genes as avirulence determinants, i.e. how do *Avr* gene products induce *R*-gene mediated resistance, as well as virulence determinants, i.e. how do *Avr* gene products contribute to virulence of the pathogen, will be discussed in detail. Four models are presented that illustrate different mechanisms underlying perception of *Avr* gene products by plants, leading either to disease susceptibility or resistance.

BACKGROUND

The first report that describes resistance of plants to fungal pathogens goes back to the end of the nineteenth century where Farrer showed that certain wheat cultivars are resistant to the rust fungus *Puccinia graminis* f. sp. *tritici* (Farrer, 1898). A few years later, in 1905, Biffen reported that wheat varieties and their progeny inherited resistance toward *Puccinia striiformis* in a Mendelian fashion (Biffen, 1905). In subsequent years, studies revealed that the resistance character is often a dominant monogenic trait, which provided the possibility to breed for resistance against pathogens. Soon after introduction of resistant plants in agriculture, however, varieties that were initially resistant to a given pathogen now became infected. In all cases, the changes were due to the appearance of new physiological races of the pathogen that were able to overcome resistance. The genetic basis of variability within a

pathogen species was first described by Johnson, who crossed two races of *P. graminis* f. sp. *tritici* and showed that inheritance of (a)virulence also followed Mendel's law (Johnson et al., 1934). Flor, working on the *Melampsora lini*-flax interaction, and Oort, working on the *Ustilago tritici*wheat interaction, were the first to present the genetic basis of specific gene-for-gene interactions between a host plant and a pathogen (Flor, 1942; Oort, 1944). These authors demonstrated that (a)virulence of physiologic races of *M. lini* and *U. tritici* is conditioned by a single pair of genes specific for each host-pathogen interaction. This gene-for-gene relationship refers to an interaction, whereby for each dominant resistance (*R*) gene in the host there is a corresponding avirulence (*Avr*) gene in the pathogen. By crossing different races of *M. lini* that are virulent on a particular flax variety to races that are avirulent, Flor showed that avirulence and virulence of pathogens is inherited as a dominant and as a recessive trait, respectively (Flor, 1958). At that time, the nature of the "mutations" leading to virulence in the flax rust fungus was unknown. Day (1957) postulated that "changing the parasite substance taking part in the primary interaction between host and pathogen would abolish defense responses leading to plant disease resistance". Indeed, recent genetic and biochemical data, obtained from various host-pathogen interactions for which a gene-for-gene relationship has been described, and which involve either viruses, bacteria, fungi, or nematodes, reveal that elicitation of defense responses is circumvented by mutations or deletions in an *Avr* gene (Nürnberger, 1999).

To explain the molecular basis of the gene-for-gene concept, various models have been proposed, which will be discussed in detail. Consistent with all models is that the product of the *Avr* gene is recognized, either directly or indirectly, by the product of the corresponding *R* gene present in the resistant plant. This recognition is often associated with a rapid local necrosis of host cells at the site of penetration, the so-called hypersensitive response (HR), which is the hallmark of gene-for-gene-based resistance and resembles programmed cell death in animals. The HR is associated with the induction of defense-related responses, including lignification, cell wall enforcement, callose deposition, accumulation of phytoalexins, and transcription of genes encoding pathogenesis-related (PR) proteins that prevent further spread of the invading pathogen.

To date, a variety of *Avr* genes has been identified to encode proteins that trigger defense responses in plants carrying the complementary *R* gene. Flor (1942) has demonstrated that *Avr*-*R* gene interactions are phenotypically epistatic over "virulence-susceptibility" gene interactions. This implies that in the presence of the complementary *R* gene, the *Avr* gene product does not provide any advantage to the pathogen, as it restricts the host range of the pathogen. Yet, although *Avr* genes have been identified as avirulence determinants, their primary function is expected to be associated with virulence rather than

with avirulence. Indeed, evidence is accumulating that *Avr* genes encode effector proteins that contribute to the establishment of a compatible interaction between pathogen and host, either by suppressing (basal) defense responses or by interacting with host-derived virulence targets. Thus, loss of the avirulence determinant, in order to overcome *R* gene-mediated resistance, might decrease the virulence of the pathogen. This implies that the most effective defense strategy for plants is to target *R* gene specificity toward *Avr* genes of which the products function to condition virulence (see this chapter).

FUNGAL (A)VIRULENCE GENES WITH GENOTYPE- AND SPECIES SPECIFICITY

Avirulence (*Avr*) genes have been discovered by virtue of the capacity of their encoded products to induce defense responses in plants carrying the corresponding resistance (*R*) gene. *Avr* genes are important determinants in the interaction between pathogen and host, as they govern host specificity. In fungus-plant interactions, fifteen *Avr* genes have thus far been cloned and demonstrated to govern either genotype- or species specificity (Table 1).

The Avr *and* Ecp *genes of* Cladosporium fulvum

Cladosporium fulvum is a biotrophic fungus that causes leaf mould of tomato plants. *C. fulvum* penetrates tomato leaves through stomata and obtains nutrients via enlarged intercellular hyphae that are in close contact with the host cells. During infection no specialized feeding structures, such as haustoria, are formed. A few weeks after penetration, when intercellular spaces are fully colonized, conidiophores emerge through stomata and numerous conidia are produced that can repeat infection of healthy tomato plants. During colonization, different proteins are secreted by *C. fulvum* into the intercellular space between the tomato mesophyll cells. Analysis of the proteins present in the apoplast of colonized tomato leaves led to the cloning of seven genes of *C. fulvum,* all of which encode elicitor proteins. Moreover, the gene encoding elicitor protein AVR2 was cloned by a functional screening of a cDNA library of *C. fulvum* that was grown *in vitro* under starvation conditions. Four elicitor proteins, AVR2, AVR4, AVR4E, and AVR9 are race-specific and trigger HR-associated defense responses in tomato plants that carry the matching *Cf* resistance gene (Joosten and De Wit, 1999). The other four elicitors, extracellular proteins ECP1, ECP2, ECP4, and ECP5, as well as ECP3, for which the encoding gene has not yet been identified, are secreted by all strains of *C. fulvum* that have been analyzed up till now (Joosten and De Wit, 1999). Individual accessions within the *Lycopersicon* genus have been identified in which these ECP proteins trigger a specific HR (Laugé et al., 2000). The

matching *R* genes, designated *Cf-ECP*s, present in these resistant individuals have not yet been introduced into commercial cultivars.

Race-specific avirulence gene *Avr2* of *C. fulvum*

Avirulence gene *Avr2* confers avirulence of *C. fulvum* on tomato plants carrying the *Cf-2* resistance gene. The *Avr2* gene was cloned based on HR induction of the encoded AVR2 protein in *Cf-2* tomato by functional screening of a cDNA library of *C. fulvum* grown *in vitro* under starvation conditions that was constructed in a binary potato virus X (PVX)-based expression vector (Takken et al., 2000a). *Avr2* encodes a cysteine-rich protein of 78 amino acids that contains a predicted signal peptide of 20 amino acids for extracellular targeting (Luderer et al., 2002b). Strains of *C. fulvum* that are virulent on *Cf-2* tomato plants carry different modifications in the open reading frame (ORF) of *Avr2* (Table 2). In addition to a variety of different single base pair deletions or insertions, all of which result in the production of truncated AVR2 proteins, one of the modifications involves a retrotransposon insertion in the *Avr2* ORF (Luderer et al., 2002b). *Cf-2*-mediated resistance has been reported to require the *Rcr3* gene (Dixon et al., 2000). *Rcr3* was isolated by positional cloning and encodes a cysteine protease that is secreted into the apoplastic space of tomato (Krüger et al., 2002). *Rcr3* was originally identified in EMS-mutagenized *Cf-2* plants that either showed a partial loss (*rcr3-1*) or a complete loss (*rcr3-3*) of *Cf-2*-mediated resistance (Dixon et al., 2000). PVX-mediated expression of *Avr2* in rcr3-1 and rcr3-3 mutant *Cf-2* plants resulted in impaired and abolished systemic HR symptoms, respectively, suggesting a role of the extracellular Rcr3 protein in perception of AVR2 by *Cf-2* plants (Luderer et al., 2002b). Thus far, no differences have been observed between the virulence of *C. fulvum* strains lacking a functional copy of *Avr2* and similar strains that are complemented with a functional genomic clone of *Avr2* (Luderer et al., 2002b).

Race-specific avirulence gene Avr4 *of C. fulvum*

The AVR4 elicitor protein is secreted into the apoplastic space of tomato as a proprotein of 135 amino acids (Joosten et al., 1994). *N*- and *C*-terminal processing by fungal and plant proteases results in a mature protein of 86 amino acids (Joosten et al., 1997). The AVR4 protein contains 8 cysteine residues, all of which are involved in intramolecular disulfide bonds (Chapter 4). Opposed to *Avr9* (see below), the *Avr4* promoter sequence does not contain nitrogen-responsive elements, indicating that *Avr4* is regulated in a different way. During pathogenesis, however, the expression profiles of both *Avr4* and *Avr9* are similar in time and space (Van den Ackerveken et al., 1994; Joosten et al., 1997). Strains of *C. fulvum*

Table 1. Cloned fungal and oomycetous avirulence genes.

Pathogen	*Avr* gene	Specificity	AVR homology	References
Cladosporium fulvum	*Avr9*	*Cf-9* tomato	Carboxypeptidase inhibitor (a)	Van den Ackerveken et al., 1992
	Avr4	*Cf-4* tomato	Chitin-binding gprotein (b)	Joosten et al., 1994; Van den Burg et al., 2003
	Avr4E	*Hcr9-4E* tomato	None	Chapter 2
	Avr2	*Cf-2* tomato	None	Luderer et al., 2002b
	Ecp1	*Cf-ECP1* tomato	Tumor necrosis factor receptor (a)	Van den Ackerveken et al., 1993; Laugé et al., 1997
	Ecp2	*Cf-ECP2* tomato	None	Van den Ackerveken et al., 1993; Laugé et al., 1997
	Ecp4	*Cf-ECP4* tomato	None	Laugé et al., 2000
	Ecp5	*Cf-ECP5* tomato	None	Laugé et al., 2000
Magnaporthe grisea	*AVR-Pita*	*Pi-ta* rice	Metalloprotease (c)	Orbach et al., 2000
	PWL1, 2	Weeping lovegrass	None	Sweigard et al., 1995
	AVR1-CO39	*CO39* rice	None	Farman et al., 2002
Rhynchosporium secalis	*NIP1*	*Rrs1* barley	Hydrophobin (a)	Rohe et al., 1995
Phytophthora parasitica	*parA1*	*Nicotiana tabacum*	None	Ricci et al., 1992
Phytophthora infestans	*Inf1*	*Nicotiana* spp.	None	Kamoun et al., 1998

(a) Structural homology, but so far no functional homology

(b) Structural homology and functional homology

(c) Sequence motif

evade *Cf-4*-mediated resistance by different single point mutations in the coding region of the *Avr4* gene (Joosten et al., 1997). These modifications either result in the production of a truncated AVR4 protein or in AVR4 isoforms that exhibit single amino acid exchanges, including cysteines (Joosten et al., 1997) (Table 2). By using PVX-mediated expression in *Cf-4* tomato, it appeared that most of these amino acid exchanges result in AVR4 isoforms that still exhibited necrosis-inducing activity, although this was significantly reduced as compared to the AVR4 wild-type protein (Joosten et al., 1997). These studies and supplementary data have demonstrated that all of these amino acid exchanges decrease protein stability, thereby circumventing specific recognition by *Cf-4* tomato plants (Chapters 2 and 4).

AVR4 shares structural homology with invertebrate chitin-binding domain proteins and binding of AVR4 to chitin oligosaccharides has been demonstrated *in vitro* (Van den Burg, et al., 2003). AVR4 also accumulates on hyphae of *C. fulvum* during growth in the apoplastic space of tomato, most likely at positions where chitin is exposed to the surface (Van den

Burg et al., 2003). Furthermore, an AVR4-specific high-affinity binding site (HABS) of fungal origin has been identified, which appeared to be heat- and proteinase K-resistant (Chapter 3). Although the latter suggests a non-proteinaceous character, AVR4 also crosslinks to a fungus-derived molecule with a molecular mass of approximately 75 kDa (Chapter 3), implying that AVR4 binds either with high affinity to a heat- and proteinase K-resistant protein or with high affinity to polysaccharides and possibly with low affinity to another fungal protein.

It appeared that, only in the presence of AVR4, the highly sensitive fungus *Trichoderma viride* is protected against the antifungal activities of plant chitinases (Van den Burg et al., 2003). The insensitivity of *C. fulvum* to plant chitinases as well as endoglucanases *in vitro* (Joosten et al., 1995), however, does not depend on the production of AVR4 by the fungus, suggesting that in this case other components protect the fungus against these hydrolases. Although not measurable *in vitro,* AVR4 might still contribute to protect *C. fulvum* against cell wall degradation during growth *in planta.*

Race-specific avirulence gene Avr4E *of C. fulvum*

Strains of *C. fulvum* that carry the *Avr4E* gene are avirulent on tomato plants carrying *Hcr9-4E* (a homologue of *Cladosporium* resistance gene *Cf-9*), which is, in addition to *Cf-4* (*Hcr9-4D*), the other functional *Cf* resistance gene present at the *Cf-4* locus (Takken et al., 1999). The *Avr4E* gene encodes a cysteine-rich protein of 101 amino acids that is secreted into the extracellular space of tomato leaves (Chapter 2). Although the *Cf-4* and *Hcr9-4E* resistance genes share a high degree of overall sequence similarity (Parniske et al., 1997), their matching *Avr* gene products do not share any sequence homology. Various strains of *C. fulvum* have been identified that evade both *Cf-4*- and *Hcr9-4E*-mediated resistance. For these strains, loss of the *Avr4* avirulence function was caused by a variety of different single point mutations in the *Avr4* allele, as mentioned earlier (Joosten et al., 1997). Loss of the *Avr4E* avirulence function appeared to be based on two different molecular mechanisms. First, strains of *C. fulvum* were identified that carry an *Avr4E* allele with two point mutations, resulting in amino acid changes $Phe^{62}Leu$ and $Met^{73}Thr$ ($AVR4E^{LT}$) (Table 2) (Chapter 2). In contrast to the AVR4 isoforms, this elicitor-inactive $AVR4E^{LT}$ protein is as stable as the wild-type AVR4E protein. It appeared that single amino acid substitution $Phe^{62}Leu$ rather than $Met^{73}Thr$ reduced the elicitor activity of AVR4E, suggesting that this single amino acid replacement $Phe^{62}Leu$ can mediate circumvention of AVR4E recognition by *Hcr9-4E* plants (Chapter 2). Single point mutations in *Avr4E*, however, which render elicitor-inactive $AVR4E^{L}$ and elicitor-active $AVR4E^{T}$, have not been identified in natural virulent and avirulent strains

of *C. fulvum*, respectively. Although we cannot exclude a possible simultaneous evolutionary event underlying the double amino acid substitution, strains of *C. fulvum* carrying *Avr4E*^{LT} most likely derived from yet unidentified avirulent strains carrying *Avr4E*^{T}.

Surprisingly, all other strains virulent on *Hcr9-4E*-containing plants carry an *Avr4E* allele that is identical to the *Avr4E* allele in avirulent strains. It appeared, however, that these strains do not secrete the AVR4E protein upon colonization of the apoplastic space of tomato (Chapter 2). Complementation of these virulent strains with a genomic *Avr4E* sequence of an avirulent strain of *C. fulvum* conferred avirulence on *Hcr9-4E* plants, suggesting that, in this case, abolished AVR4E expression results in circumvention of *Hcr9-4E*-mediated resistance. Indeed, no *Avr4E* transcripts could be detected when northern blot analysis was performed on RNA isolated from a compatible interaction between these strains and tomato. Whether recombination events or (transposon) insertions within the promoter sequence of *Avr4E* cause abolished AVR4E expression still needs to be elucidated.

Race-specific avirulence gene *Avr9* of *C. fulvum*

Avr9, which is the first fungal *Avr* gene that has been cloned and characterized, encodes a precursor protein of 63 amino acids that contains a 23-amino acid signal sequence (Van Kan et al., 1991). Upon secretion into the apoplast, AVR9 is further processed at the *N*-terminus by fungal and plant proteases into a mature protein of 28 amino acids, six of which are cysteines. The three-dimensional structure of AVR9, elucidated by ^{1}H-NMR, revealed that the protein contains three antiparallel â-strands that are interconnected by three disulfide bridges (Van den Hooven et al., 2001). The AVR9 protein, which contains a cystine knot, is structurally most related to potato carboxypeptidase inhibitor (CPI) (Van den Hooven et al., 2001). AVR9, however, does not have amino acid residues identical to those located at known CPI-inhibitory sites and thus far no protease-inhibiting activity could be detected. Structural analysis revealed that all six cysteine residues present in AVR9 are essential for its structure and necrosis-inducing activity (Kooman-Gersmann et al., 1997; Van den Hooven et al., 2001). In addition, residue Phe^{21}, present in the solvent-exposed hydrophobic â-loop region, is also essential for the necrosis-inducing activity of AVR9 (Kooman-Gersmann et al., 1997). Moreover, when applied to transgenic *Cf-9* tobacco cell suspensions, AVR9 mutant peptide carrying Phe^{21}Ala is incapable of inducing medium alkalization, whereas its capacity to induce an oxidative burst was reduced (De Jong et al., 2000). Virulence of *C. fulvum* strains on *Cf-9* plants appeared to be the result of a deletion of the entire *Avr9* gene (Table 2). Moreover, disruption of *Avr9*

by homologous recombination in *C. fulvum* strains that are normally avirulent on *Cf-9* plants did not affect *in vitro* growth or virulence of the fungus on susceptible tomato plants, suggesting that *Avr9* is dispensable for full virulence (Marmeisse et al., 1993). Although dispensable, the expression of *Avr9* is induced under nitrogen-limiting conditions *in vitro* (Van den Ackerveken et al., 1994; Pérez-García et al., 2001), which suggests that AVR9 might be involved in the nitrogen metabolism of the fungus. Pérez-García et al. (2001) identified a gene in *C. fulvum*, designated *Nrf1*, which has a strong similarity to nitrogen regulatory proteins of *Aspergillus nidulans.* Although *Nrf1*-deficient strains do not express *Avr9* under nitrogen starvation conditions *in vitro,* these strains are still avirulent on *Cf-9* tomato plants, suggesting that NRF1 is a major, yet not the only, positive regulator of *Avr9* expression (Pérez-García et al., 2001).

Non-race-specific extracellular protein (Ecp) *genes of C. fulvum*

Four genes encoding extracellular proteins ECP1, ECP2, ECP4, and ECP5 have been cloned (Van den Ackerveken et al., 1993; Laugé et al., 2000). These *Ecp* genes all encode cysteine-rich proteins that are abundantly secreted by all strains of *C. fulvum* during colonization of tomato leaves. These proteins do neither share sequence homology with each other nor with any sequences present in the database. Although the even number of cysteine residues present in the ECPs suggests that these residues contribute to protein stability, some (as demonstrated for ECP1 and ECP2) appear not to be involved in intramolecular disulfide bonds (Luderer et al., 2002a). As found for *Avr4* and *Avr9,* transcription of both *Ecp1* and *Ecp2* is strongly induced *in planta* (Wubben et al., 1994), indicating that plant-derived signals are required for the induction of both *Avr* and *Ecp* gene expression. Tomato accessions that develop a HR upon inoculation with recombinant PVX expressing *Ecp2* have been identified (Laugé et al., 1998b). The responding accessions all carry a single dominant gene, designated *Cf-ECP2* gene, and show HR-associated resistance toward ECP2-producing strains of *C. fulvum* (Laugé et al., 1998b). ECP1, ECP3, ECP4, and ECP5 have also been shown to act as elicitors of HR on tomato accessions and wild *Lycopersicon* plants that are resistant toward *C. fulvum*, most likely through recognition of the corresponding secreted ECP (Laugé et al., 2000). Opposed to the *Avr* genes, no modifications have thus far been found in the *Ecp* genes of naturally occurring strains of *C. fulvum*. This might be due to a lack of selection pressure on the pathogen to overcome *Cf-ECP*-mediated resistance, as the *Cf-ECPs* have not yet been introduced in commercial cultivars. On the other hand, as all strains of *C. fulvum* analyzed so far secrete the ECPs, disruption or modification of the encoding genes is thought to cause reduced virulence of the

fungus on tomato. Indeed, the *Ecp2* gene appears to be required for colonization and sporulation of *C. fulvum* on mature tomato plants (Laugé et al., 1997). Moreover, *Ecp1*-deficient strains fail to sporulate as abundantly as the wild-type strain on mature tomato plants (Laugé et al., 1997). This implies that ECP1 and ECP2 are both required for full virulence of *C. fulvum* on tomato. In addition, both *Ecp1*- and *Ecp2*-deficient strains induce plant defense-associated responses more quickly and to higher levels than wild-type strains, suggesting that both ECPs are involved in suppression of host defense-associated responses during colonization (Laugé et al., 1997). Based on this observation, an interesting parallel can be drawn with mammalian systems, in which viruses have been reported to produce extracellular suppressors of host defense responses (Laugé et al., 1997). Interestingly, ECP1 shares structural homology (based on the spacing of the cysteine residues) with a viral T2 suppressor protein as well as with the family of tumor-necrosis factor receptors (TNFRs) (Laugé et al., 1997). The T2 suppressor protein compromises the establishment of host defense responses by interacting with mediators of the immune system (tumor necrosis factors), thereby preventing its binding to endogenous TNFRs. Furthermore, a putative receptor-like kinase has been identified in plants that shares structural homology with the TNFR family. One possibility could be that ECP1 competitively inhibits the binding of defense signaling molecules to this plant receptor protein, thereby suppressing the induction of host defense responses.

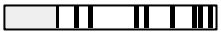
The Avr *genes of* Magnaporthe grisea

The filamentous ascomycete *Magnaporthe grisea* is the causal agent of blast disease on many species of the grass family, such as rice. *M. grisea* initiates infection by a germinating conidium that quickly differentiates into a specialized cell, the appressorium. Once mature, the melanized appressorium generates enormous hydrostatic pressure that forces a narrow penetration peg through the plant cuticle and epidermal cell wall. After penetration, the fungus grows intracellularly and produces sporulating lesions within five to seven days.

Genotype-specific Avr *genes of* M. grisea

Strains of *M. grisea* that carry the *Avr-Pita* (*AVR2-YAMO)* gene are avirulent on rice cultivars that carry the corresponding *R* gene *Pi-ta* (Orbach et al., 2000). The *Avr-Pita* gene is located very close to the telomere of chromosome 3 and encodes a predictedpolypeptide of 223 amino acids. AVR-Pita exhibits substantial similarity to NPII, a neutral zinc

Table 2. Overview of mutations identified in the open reading frames of *Avr2*, *Avr4*, *Avr4E*, and *Avr9* of *Cladosporium fulvum* strains that are virulent on tomato plants that carry *Cf-2, Cf-4*, *Hcr9-4E* or *Cf-9*, respectively.

Mutations in *Avr2*	Codon position in ORF	Mutation in AVR2	Predicted protein*
Wild-type	-	No mutation	
C to T (stop)	66	Frame shift/stop	
T	72	Frame shift	
C	24	Frame shift/stop	
A	23	Frame shift/stop	
+A	40	Frame shift/stop	
+A	23	Frame shift/stop	
+ Transposon	19	Insertion of 5 kB	

Mutations in *Avr4*	Codon position in ORF	Mutation in AVR4	Predicted protein
Wild-type	-	No mutation	
C	42	Frameshift	
G to T	64	Cys-64-Tyr	
C to T	66	Thr-66-Ile	
T to C	67	Tyr-67-His	
G to T	70	Cys-70-Tyr	
G to T	109	Cys-109-Tyr	

Mutations in *Avr4E*	Codon position in ORF	Mutation in AVR4E	Predicted protein
Wild-type	-	No mutation	
T to C; T to C	82 and 93	Phe-82-Leu; Met-93-Thr	

Mutation in *Avr9*			Predicted Protein
Wild-type	-	No mutation	
Deletion of ORF		No protein	No protein

*) The speckled areas in the horizontal bars represent the signal peptide of the AVR proteins; open areas represent the mature part of the AVR protein; hatched areas represent the amino acid sequence encoded that follows the frameshift mutation in *Avr2* and *Avr4*. The cysteine residues are indicated as vertical lines and amino acid substitutions as dotted vertical lines. The black areas represent the amino acid sequence that is removed by N- and C-terminal processing.

metalloprotease from *Aspergillus oryzae*. Based on this homology, the *N*-terminus of AVR-Pita was predicted to be further processed to an active form of 176 amino acids. This AVR-Pita$_{176}$ protein, but not the intact AVR-Pita$_{223}$ protein and AVR-Pita$_{166}$ (which has an additional deletion at the *N*-terminus), triggers the *Pi-ta*-dependent HR when produced inside rice cells by transient expression (Jia et al., 2000). In the region that corresponds to the consensus zinc binding domain of neutral zinc metalloproteases, residue Glu-177 of AVR-Pita$_{223}$ (i.e. Glu-130 of AVR-Pita$_{176}$) is predicted to be essential for metalloprotease activity. Interestingly, replacement of this Glu residue by Asp, as found in spontaneous gain of virulence mutants, abolishes the HR-inducing ability of AVR-Pita$_{176}$ (Jia et al., 2000; Orbach et al., 2000). This implies that the protease activity of *AVR-Pita*, although not yet biochemically demonstrated, plays an essential role in avirulence (Orbach et al., 2000). The majority of spontaneous virulent mutants of *M. grisea* that carry deletions ranging from 100 bp up to 10 Kb, which is consistent with the genetic instability observed for genes that are located at a telomere (Orbach et al., 2000). In addition to point mutations and deletions, gain of virulence on *Pi-ta* rice cultivars was also mediated by an insertion of a pot3 transposon into the promoter of *AVR-Pita* (Kang et al., 2001). Despite its putative metalloprotease activity, no role in virulence could yet be assigned to AVR-Pita.

The *AVR1-CO39* gene of *M. grisea* has been identified as the minimal (1.05 kB) fragment that confers avirulence on rice cultivar CO39 (Farman et al., 2002). Only a small number of rice-infecting *M. grisae* isolates from the Philippines, however, are avirulent on this cultivar. While most virulent isolates lack the entire *AVR1-CO39* locus, it appeared that in some cases complex genomic rearrangements have occurred at the *AVR1-CO39* locus, each of which resulting in non-functional alleles (Farman et al., 2002).

Species-specificity conferred by PWL *genes of M. grisea*

The *PWL2* (for Pathogenicity toward Weeping Lovegrass) gene of *M. grisea* determines host-species specificity. Strains of the fungus expressing *PWL2* are avirulent on weeping lovegrass, but virulent on rice and barley (Sweigard et al., 1995). *PWL2* encodes a glycine-rich protein of 145 amino acids with a putative signal peptide for extracellular targeting. Analysis of spontaneous virulent mutants on weeping lovegrass revealed that the *PWL2* allele is genetically unstable, although it is not located at a telomere (Sweigard et al., 1995). As found for the *avr-pita*-deficient mutants, spontaneous deletion of *PWL2* had no apparent effect on virulence under laboratory conditions. Strains of *M. grisea* also evade PWL2 recognition by a single base pair change that results in the creation of a putative *N*-

glycosylation site. This PWL2 mutant protein exhibits reduced elicitor-activity either due to glycosylation or due to the amino acid change itself.

The *PWL2* gene is a member of a rapidly evolving gene family of which the homologue *PWL1* and the allelic *PWL3/PWL4* genes map at different chromosomal locations (Kang et al., 1995). The PWL2 protein is 75 percent identical to the PWL1 protein, and 51- and 57 percent identical to the PWL3 and PWL4 proteins, respectively. Opposed to *PWL1* and *PWL2,* the *PWL3* and *PWL4* genes are non-functional *Avr* genes, as they do not confer avirulence on weeping lovegrass. In contrast to *PWL3, PWL4* becomes functional in preventing infection of weeping lovegrass when its expression is driven by either the *PWL1* or the *PWL2* promoter (Kang et al., 1995). This indicates that *PWL4* encodes a functional AVR protein, which is not recognized by weeping lovegrass due to lack of expression of the gene.

The Avr *genes of* Rhynchosporium secalis

The fungus *Rhynchosporium secalis* is known as the causal agent of leaf scald on barley, rye and other grasses. *R. secalis* initiates infection by penetrating the cuticle, followed by extracellular growth of hyphae between the cuticle and the outer epidermal cell walls. The fungus develops an extensive subcuticular stroma, causes an early collapse of a few epidermal cells and the underlying mesophyll cells, and finally starts to sporulate. Amongst the secreted proteins in culture filtrates of *R. secalis,* a class of necrosis-inducing proteins, NIPs, has been identified that induce necrosis in certain barley cultivars and other cereals (Wevelsiep et al., 1993). The phytotoxicity of these NIPs, which is associated with lesion-development, appeared to be based on their stimulatory effect on the plant plasmalemma H^+-ATPase, probably in order to release plant nutrients (Wevelsiep et al., 1993).

Strains of *R. secalis* that secrete NIP1 are unable to grow on barley cultivars that carry the *Rrs1* gene. *Rrs1*-mediated resistance is not associated with a rapid HR, but with the accumulation of mRNAs encoding peroxidase and PR proteins of the PR-5 class (Rohe et al., 1995). The *NIP1* gene encodes a secreted elicitor-active protein of 60 amino acids, 10 of which are cysteine residues (Rohe et al., 1995). The three-dimensional structure of NIP1 revealed that all 10 cysteines form intramolecular disulfide bonds, providing stability to the protein (Van 't Slot, unpublished data). The spacing pattern of the first eight cysteine residues in NIP1 (-C-CC-C-C-CC-C-) has also been found in another class of fungal proteins, the hydrophobins. The partially resolved disulfide bond pattern of the *Ophiostoma ulmi* hydrophobin, however, differs from that of NIP1, suggesting that NIP1 is not functionally

related to the hydrophobins. Thus far, no structural homology has been found between NIP1 and other proteins (Van 't Slot, unpublished data). Several avirulent races of *R. secalis* carry three amino acid changes in the *NIP1* gene product (NIP1 type II). Despite the fact that elicitor activity is reduced, these NIP1 type II proteins still confer avirulence and still exhibit toxicity. Two elicitor-inactive NIP1 proteins (NIP1 type III and IV proteins) have been identified to carry two different, additional amino acid substitutions (Rohe et al., 1995). Moreover, virulence of *R. secalis* on *Rrs1* barley plants is accomplished by deletion of the entire *NIP1* gene (Rohe et al., 1995). These strains lacking *NIP1* are less virulent on susceptible barley cultivars than those carrying *NIP1*, demonstrating that NIP1 plays a role in virulence of *R. secalis* and that toxic activity of NIP1 type III and IV is retained (Rohe et al., 1995).

The genes encoding elicitins of* Phytophthora *spp

Oomycetous plant pathogens, such as *Phytophthora* spp., downy mildews and *Pythium* spp., cause devastating diseases on numerous crops and ornamental plants. In the middle of the nineteenth century, *Phytophthora infestans* destroyed potato crops in Ireland, which resulted in starvation and decimation of the population. Despite the fact that many *R* genes have been incorporated into potato through traditional breeding strategies, the late-blight pathogen has remained a continuous threat for potato growers world-wide because of its adaptive abilities.

Although oomycetes exhibit filamentous growth, they share little taxonomic affinity to filamentous fungi and are more closely related to eukaryotic algae (Kamoun et al., 1999b). The disease cycle of oomycetes starts when zoospores encyst and germinate on root or leaf surfaces. In some species, sporangia germinate directly. Germ tubes penetrate the epidermal cell layer, secondary hyphae expand through the intercellular space to neighboring cells, and in some cases feeding structures are formed inside the mesophyll cells. The major defense reaction in resistant plants to many *Phytophthora* and downy mildew species is associated with a HR. Partial resistance to *Pythium* , however, appeared to be mediated by physical barriers rather than by a HR (Kamoun et al., 1999b).

P. infestans, as well as other *Phytophthora* and *Pythium* species, produce extracellular proteins of 10 kDa, termed elicitins, which contain three highly conserved disulfide bridges (Huet et al., 1995; Boissy et al., 1996). It has been demonstrated that elicitins bind to sterols and mediate their transfer between micelles and artificial phospholipid membranes (Mikes et al., 1998). As *Phytophthora* species do not synthesize sterols themselves, elicitins might contribute to the assimilation and growth of the oomycete. Elicitins induce non-genotype

specific defense-associated responses, including a HR, in plants of the genus *Nicotiana* (i.e. Solanaceae) and in some cultivars of radish, turnip, and rape (i.e. Cruciferae).

Species-specific gene parA1 *of* Phytophthora parasitica

In *Phytophthora parasitica*, the absence of elicitin production correlated with high virulence on tobacco. Although elicitins are encoded by a multigene family, it appeared that *parA1* is the main elicitin-encoding gene expressed *in vitro* and *in planta* by *P. parasitica*. The *parA1* gene was cloned from *Phytophthora parasitica* and encodes a secreted protein parasiticein of 98 amino acids, of which 6 are cysteine residues (Ricci et al., 1992). The *parA1* gene has been proposed to act as a species-specific *Avr* gene, as it triggers HR-mediated resistance toward *P. parasitica* in *Nicotiana tabacum* (tobacco). Elicitin-producing *P. parasitica* isolates have been demonstrated to cause disease on tobacco, but not on tomato, upon down-regulation of parA1 expression (Colas et al., 2001). It appeared that this down-regulation event relies on a mechanism that is dependent on the *P. parasitica* genotype rather than the host plant (Colas et al., 2001).

Genotype-specific gene inf1 *of* Phytophthora infestans

The *inf1* gene of *P. infestans* encoding infestin was cloned by screening a cDNA library of a compatible interaction between *P. infestans* and potato with a *parA1* gene fragment (Kamoun et al., 1997). While high levels of *inf1* transcripts are observed in mycelium grown *in vitro*, the expression of *inf1* is down-regulated *in planta* (Kamoun et al., 1997). Opposed to *parA1,* down-regulation of *inf1* expression is not required to evade plant defense responses, as the host plant potato does not respond to INF1 (Kamoun et al., 1997). In leaves of non-host *Nicotiana* plants, however, injection of INF1 induces a specific HR. To determine whether INF1 plays a role in non-host resistance, members of the *Nicotiana* family were inoculated with *inf1*-deficient *P. infestans* strains. These *inf1*-deficient strains are able to cause disease when inoculated on leaves of *N. benthamiana*, whereas on other *Nicotiana* species, such as *N. tabacum* (tobacco), however, these *inf1*-deficient strains are still avirulent (Kamoun et al., 1998). This demonstrates that INF1 confers non-host resistance toward *P. infestans* in *N. benthamiana,* but is not the main determinant of non-host resistance to *P. infestans* in other *Nicotiana* species. Putative additional candidates that confer avirulence on tobacco are the products of the *inf2A* and *inf2B* genes of *P. infestans*, which also induce a HR when injected into *N. tabacum* leaves (Kamoun et al., 1998).

PATHOGENS HAVE EVOLVED MECHANISMS TO COUNTERACT PLANT DEFENSE

As describe above, plant pathogens evade *R* gene-mediated resistance by modification of the elicitor proteins either by mutations in, or deletion of, the *Avr* genes or by (down)-regulation of *Avr* gene expression. Yet, when the circumvention of elicitor detection fails, or when the elicitor component is essential for virulence, pathogens require mechanisms to subvert the induced plant defense responses. Indeed, plant pathogens counteract plant defenses by secreting enzymes that detoxify defense compounds, including phytoalexins, or use ATP-binding cassette (ABC)-transporters to mediate the efflux of toxic compounds (as reviewed by Idnurm and Howlett, 2001). Moreover, some bacterial pathogens interfere with *R* gene-mediated resistance by secreting proteins that "mask" the presence of a particular AVR effector protein (Ritter and Dangl, 1996).

Recent studies revealed that some pathogenic fungi have evolved counter-defense mechanisms that enable the suppression of plant defense responses. This mechanism involves a class of proteins, termed glucanase inhibitor proteins (GIPs), which are secreted by *Phytophthora sojae* f. sp. *glycines* and which inhibit the endoglucanase (EnGL) activity of its host soybean (Rose et al., 2002). Sequences homologous to *GIPs* have been identified in genomic DNA of other *Phytophthora* species, while several other plant pathogenic fungi do not exhibit related sequences (Rose et al., 2002). GIPs are homologous to the trypsin class of serine proteases, but do not exhibit proteolytic activity. The basis of endoglucanase inhibition by GIPs involves the formation of a stable complex. This association appeared to be specific, as GIP1 inhibits soybean endoglucanase-A (EnGL-A) but not EnGL-B. GIPs also suppress the release of elicitor-active oligoglucosides from *P. sojae* mycelial walls *in vitro* and during pathogenesis *in vivo* (Rose et al., 2002). Thus, GIPs suppress cell wall disassembly by inhibiting endoglucanases, and suppress the activation of defense-associated responses by inhibiting the release of oligoglucoside elicitors (Rose et al., 2002).

The *CgDN3* gene of the hemi-biotrophic pathogen *Colletotrichum gloeosporioides*, which encodes an extracellular protein of 54 amino acids, is induced under nitrogen starvation conditions at the early stage of infection on tropical pasture legume (Stephenson et al., 2000). On intact leaves of a normally compatible host, *CgDN3*-disrupted mutants were nonpathogenic and elicited a hypersensitive-like response, the latter of which may be part of a basal defense reaction toward *C. gloeosporioides* (Stephenson et al., 2000). When applied to wound sites, however, these mutants were able to grow necrotropically on host leaves and form necrotic spreading lesions. It was therefore suggested that at the early stages of the primary infection process, CgDN3 is required for pathogenicity and functions to suppress the hypersensitive-like response (Stephenson et al., 2000). Opposed to the *C. fulvum* ECP1

and ECP2 elicitors that also suppress host defense responses (Laugé et al., 1997), no elicitor function has been assigned to CgDN3.

PLANT GENES THAT CONFER RESISTANCE TOWARD FUNGI AND OOMYCETES

R genes are very abundant in plant genomes and in most cases they belong to tightly linked gene families. The *R* genes that have been characterized can be divided in six classes of proteins (reviewed by Takken and Joosten, 2000b) (Table 3). Three of these classes contain leucine-rich repeats (LRRs), of which the class of nucleotide-binding site (NB)-LRRs proteins is the most abundant. The NB-LRR class can be further subdivided, based on the deduced N-terminal features of the R protein. The N-terminus of one subclass contains a TIR domain, which has homology to the *Drosophila Toll* and mammalian *Interleukin-1* receptors, while the other contains putative coiled-coil (CC) domains. The TIR-NB-LRR and CC-NB-LRR proteins confer resistance to a broad range of pathogens, including viruses, bacteria, fungi, oomycetes, nematodes, and insects.

The other two classes of LRR proteins involve the LRR-transmembrane-anchored (LRR-TM) proteins and the LRR-TM-kinase proteins. The LRR-TM proteins have been demonstrated to provide resistance toward fungi and nematodes, whereas the LRR-TM-kinase (i.e. Xa-21) confers bacterial (*Xanthomonas oryzae* pv. *oryzae*) resistance.

Members of the fourth class of *R* genes represent protein kinases, which, in the case of *Pto*, confer resistance toward *Pseudomonas syringae* pv. *tomato* carrying *AvrPto.* To date, *R* genes against fungi that encode protein kinases have not been identified, yet, their involvement in defense-signaling pathways leading to fungal disease resistance cannot be excluded.

The fifth class of *R* genes is represented by *RPW8,* a small, putative membrane protein with a possible cytoplasmic coiled-coil domain that confers downy mildew (*Erysiphe cichoracearum*) resistance in *Arabidopsis* (Xiao et al., 2001b).

The tomato verticillium wilt *Ve* resistance genes from tomato represent the sixth class of *R* genes (Kawchuk et al., 2001). The two closely linked *Ve1* and *Ve2* genes, which products might recognize different ligands, encode TM-surface glycoproteins having an extracellular LRR domain, endocytosis-like signals and leucine zipper (LZ) or Pro-Glu-Ser-Thr (PEST) sequences. The LZ can facilitate dimerization of proteins through the formation of CC structures, while PEST sequences are often involved in ubiquitination, internalization, and degradation of proteins. Receptor-mediated endocytosis could provide a mechanism through which cells capture ligands and remove signaling receptors from the cell surfaces (Kawchuk et al., 2001).

Table 3. R proteins identified in gene-for-gene interactions conferring resistance toward fungi and oomycetes, classified based on homologous structural domains. TIR, Toll/interleukin 1 receptor-like domain; NB, nucleotide binding site; LRR/LRD, leucine-rich repeat/domain; CC, coiled-coil; TM, transmembrane domain; PEST, Pro-Glu-Ser-Thr sequences.

R gene	Pathosystem	Structure	Matching *Avr* gene	References
L6, M, N, P	Flax/ *Melampsora lini*	TIR-NB-LRR	Unknown	Islam and Mayo, 1990
RPP1, 10, 14	*Arabidopsis/ Peronospora parasitica*	TIR-NB-LRR	Unknown	Botella et al., 1998
RPP4, 5	*Arabidopsis/ Peronospora parasitica*	TIR-NB-LRR	Unknown	Van der Biezen et al., 2002
RPP8	*Arabidopsis/ Peronospora parasitica*	CC-NB-LRR	Unknown	McDowell et al., 1998
RPP13	*Arabidopsis/ Peronospora parasitica*	CC-NB-LRR	Unknown	Bittner-Eddy et al., 2000
Mla	Barley/ *Erysiphe graminis* f.sp. *hordei*	CC-NB-LRR	Unknown	Halterman et al., 2001
R1	Potato/ *Phytophthora infestans*	CC-NB-LRR	Unknown	Ballvora et al., 2002
Dm3	Lettuce/ *Bremia lactucae*	NB-LRR	Unknown	Anderson et al., 1996
I2	Tomato/ *Fusarium oxysporum* f.sp. *lycopersici*	NB-LRR	Unknown	Ori et al., 1997
Rp1	Maize/ *Puccinia sorghi*	NB-LRR	Unknown	Richter et al., 1995
Pi-ta	Rice/ *Magnaporthe grisea*	NB-LRD	*Avr-Pita*	Bryan et al., 2000
Cf-2	Tomato/ *Cladosporium fulvum*	LRR-TM	*Avr2*	Dixon et al., 1996
Cf-4	Tomato/ *Cladosporium fulvum*	LRR-TM	*Avr4*	Thomas et al., 1997
Hcr9-4E	Tomato/ *Cladosporium fulvum*	LRR-TM	*Avr4E*	Takken et al., 1999
Cf-5	Tomato/ *Cladosporium fulvum*	LRR-TM	Unknown	Dixon et al., 1998
Cf-9	Tomato/ *Cladosporium fulvum*	LRR-TM	*Avr9*	Jones et al., 1994
Rpw8	*Arabidopsis/ Erysiphe cichoracearum*	CC-TM	Unknown	Xiao et al., 2001b
Ve1, Ve2	Tomato/ *Verticillium dahliae*	CC-LRR-TM-PEST	Unknown	Kawchuk et al., 2001

The NB-LRR class of R proteins

In flax, NB-LRR proteins have been identified that mediate recognition of 31 different rust (*Melampsora lini)* strains. These different resistance specificities are distributed among five polymorphic loci, *K, L, M, N,* and *P* (Islam and Mayo, 1990). Comparative sequence analysis of the R proteins encoded by the flax *L* alleles has demonstrated that both LRR and TIR domains play a role in determining resistance specificities (Luck et al., 2000). Flax *R* genes confer only resistance to those strains of *M. lini* that carry the corresponding *Avr* gene, however, none of the 31 genetically defined *Avr* genes has been cloned yet.

In *Arabidopsis*, several *R* gene loci have been identified that confer resistance toward strains of the oomycetous pathogen *Peronospora parasitica*. These *RPP* loci comprise genes that encode TIR-NB-LRR proteins (RPP1, 4, 5, 10, and 14) and CC-NB-LRR proteins

(RPP8 and RPP13) (Botella et al., 1998; Van der Biezen et al., 2002; Bittner-Eddy et al., 2000; McDowell et al. 1998). The *RPP4* gene of the *Arabidopsis* landrace Columbia (Col) is an orthologue of the *RPP5* gene of Landsberg *erecta* (L*er*). *RPP4* confers resistance to *P. parasitica* races Emoy2 and Emwa1, while *RPP5* confers resistance toward these races as well as toward *P. parasitica* race Noco2 (Van der Biezen et al., 2002). These strains of *P. parasitica* might carry two distinct, yet not identified, *Avr* determinants, i.e. *AvrRPP4* and *AvrRPP5* that are recognized by RPP4 and RPP5, respectively. *RPP4* and *RPP5*, on the other hand, could also have overlapping specificities, whereby AvrRPP4, but not AvrRPP5, is recognized by both RPP4 and RPP5.

Other *R* genes that belong to the NB-LRR class, which are also members of complex resistance loci, confer multiple resistance specificities toward fungal pathogens as well. In barley, the *Mla* locus confers resistance against various races of powdery mildew (*Erysiphe graminis* f. sp. *hordei*) on a gene-for-gene basis (Halterman et al., 2001). Moreover, the *Rp1* rust (*Puccinia sorghi*) locus in maize and the *Dm3* downy mildew (*Bremia lactucae*) locus in lettuce all contain multiple genetically linked resistance specificities (Richter et al., 1995; Anderson et al., 1996). For all of these gene-for-gene interactions no matching *Avr* genes have thus far been characterized.

The rice blast gene *Pi-ta* confers gene-for-gene-based resistance against strains of *M. grisea* that express *AVR-Pita. Pi-ta* encodes a putative cytoplasmic receptor and is a member of the NB-receptor class of *R* genes. Pi-ta lacks an N-terminal TIR or CC domain and the C-terminal leucine-rich domain (LRD) lacks the characteristic LRR motif found in other proteins of the NB-LRR class. The predicted protein encoded by *Pi-ta* present in resistant rice varieties differs by only one amino acid, located at position 918, from the protein encoded by susceptible rice varieties (Bryan et al., 2000). Moreover, it appeared that Pi-ta protein with alanine-918, but not Pi-ta with serine-918, interacts with AVR-Pita in the cytoplasm of rice cells to induce resistance responses.

The TM-LRR class of R proteins

Another class of *R* genes includes the *Cf* genes of tomato, which confer resistance to strains of *C. fulvum* carrying the corresponding *Avr* gene (Table 3). The *Cf* genes encode proteins with a predicted signal peptide for extracellular targeting, a LRR region, a TM domain and a short cytoplasmic tail (Jones and Jones, 1996; Joosten and De Wit, 1999). The *Cf* genes are members of multigene families and have been designated *Hcr2* or *Hcr9* (for homologues of *Cladosporium* resistance genes *Cf-2* and *Cf-9,* respectively). Two nearly identical *Cf-2* genes (*Cf-2.1* and *Cf-2.2*) and *Cf-5* (*Hcr2-5C*) confer resistance toward *C.*

fulvum strains through recognition of the *Avr2* and *Avr5* gene products, respectively (Dixon et al., 1996 and 1998). The *Cf-4* and *Cf-9* gene clusters each consist of five *Hcr9* homologues, of which *Hcr9-4D* (i.e. *Cf-4*), *Hcr9-4E* and *Hcr9-9C* (i.e. *Cf-9*) gene products specifically recognize AVR4, AVR4E and AVR9, respectively (Thomas et al., 1997; Jones et al., 1994; Takken et al., 1999).

PERCEPTION OF AVR *GENE PRODUCTS BY RESISTANT PLANT GENOTYPES*

In the absence of the corresponding *R* gene, many *Avr* genes have been demonstrated to provide a selective advantage to the pathogen (Laugé and De Wit, 1998c; White et al., 2000; Staskawicz et al., 2001; Bonas and Lahaye, 2002). This, together with the maintenance of *Avr* genes within pathogen populations, implies that the primary function of *Avr* genes is to confer virulence to the pathogen. Although for most *Avr* gene products no biological function has yet been defined, it appears that in colonized plant tissue AVR proteins co-localize with various host virulence targets. Together with the fact that proper subcellular targeting is essential for the avirulence activity of AVRs, this implies that AVR proteins, virulence targets and R proteins are possibly part of one complex (Van der Hoorn et al., 2002). Four models have been proposed addressing the question how and where AVR proteins are recognized by resistant plant genotypes (Fig. 1). These models will be discussed in detail in the following section.

Direct perception of AVR proteins

This model reflects the most simple interpretation of Flor's gene-for-gene hypothesis; a classical receptor-ligand model that predicts a direct interaction between *Avr* and *R* gene products (Fig. 1). Thus far, direct physical interaction has only been demonstrated for Pto from tomato and AvrPto from *Pseudomonas syringae* (Tang et al., 1996), and for Pi-ta from rice and AVR-Pita from *M. grisae* (Jia et al., 2000). The cytoplasmic localization of both Pto and Pi-ta is consistent with the observation that *AvrPto* and *Avr-Pita* induce a HR when expressed inside plant cells (Scofield et al., 1996; Jia et al., 2000). Pto is a Ser/Thr kinase that interacts with and phosphorylates a second Ser/Thr kinase, Pti1, and several defense-related transcription factors, such as Pti4, Pti5, and Pti6 (Martin et al., 1993; Xiao et al., 2001a). The most straightforward explanation for induction of plant defense responses would be that binding of AvrPto to Pto leads to a conformational change of the kinase protein that in turn triggers downstream defense signaling pathways. Pto function, however, requires *Prf*, a NB-LRR-encoding gene (Salmeron et al., 1996). Therefore, the "co-receptor"

and/or the “guard” model (see below) was put forward to rationalize the mechanism of AvrPto-induced defense activation (Fig. 1). AVR-Pita recognition, on the other hand, is mediated by direct interaction with the LRD of Pi-ta (Bryan et al., 2000; Jia et al., 2000). It appeared that the putative metalloprotease activity of AVR-Pita is required for its direct interaction with Pi-ta (Jia et al., 2000), suggesting a protease-dependent defense elicitation model (Fig. 1). Possibly, Pi-ta contains protease cleavage sites, which upon proteolytic processing renders an active form either by a conformational change or by the release of elicitor peptide(s) that trigger(s) defense responses.

Indirect perception of AVR proteins

In addition to direct perception of AVR proteins by R proteins, three models have been proposed that postulate an indirect interaction between *Avr* and *R* gene products to take place. These models include enzymatic- or protease-dependent defense elicitation, or the involvement of a “co-receptor” or a “guard” protein in triggering defense responses.

Protease-dependent defense elicitation model

Rcr3, a gene required for *Cf-2*-mediated resistance toward *C. fulvum* strains carrying *Avr2*, has recently been cloned and encodes a tomato cysteine endoprotease (Krüger et al., 2002). The fact that Rcr3 is secreted into the apoplastic space is consistent with the extracellular localization of AVR2 and Cf-2. Several protease-dependent mechanisms underlying the *Cf-2*-mediated resistance can be envisaged, whereby Rcr3 most likely functions upstream of Cf-2. Rcr3 might process AVR2 to generate a mature ligand, or Rcr3 might degrade AVR2, thereby releasing active elicitor peptides that interact with the extracellular LRR of Cf-2. This would imply that not AVR2 itself but rather a protease-dependent signal triggers *Cf-2*-mediated resistance. Another possibility is that AVR2 forms a complex with Rcr3, which subsequently triggers *Cf-2*-dependent downstream signaling pathways that lead to disease resistance. Rcr3, on the other hand, might also be part of a basal defense mechanism of the plant that upon inhibition by AVR2 turns on *Cf-2* mediated defense responses (see below; “guard hypothesis”).

The “co-receptor” model

Most *R* genes encode proteins that carry LRR domains. These LRRs, located either intracellular (in the case of most NB-LRR proteins) or extracellular (in the case of LRR-TM

proteins), have been implicated to function in protein-protein interactions (Jones and Jones, 1996). NB-LRR proteins often function together with Ser/Thr kinases to trigger *R*-mediated signal transduction pathways, as demonstrated for Pto kinase, which requires the NB-LRR protein Prf (Salmeron et al., 1996), as well as for the NB-LRR protein RPS5, which requires PBS1 kinase (Swiderski and Innes, 2001). Moreover, in the development of shoot apical meristem, CLV1, a receptor-like protein kinase with extracellular LRRs, and CLV2, a protein with predicted extracellular LRRs and a short cytoplasmic domain, function together to recognize an extracellular peptide encoded by *CLV3* (Rojo et al., 2002). Interestingly, the *Cf* gene family is similar to *CLV2* in that it encodes plasma membrane anchored proteins with predicted extracellular LRRs and short cytoplasmic regions that lack any obvious downstream signaling domains. Recently, it has been demonstrated that both Cf-4 and Cf-9 are part of a heteromultimeric membrane-associated complex of approximately 420 kDa (Rivas et al., 2002a and 2002b). Based on the analogy between the two systems, it could be possible that a component homologous to CLV1, i.e. a receptor-like kinase, is required for *Cf*-mediated perception of extracellular AVR proteins (Joosten and De Wit, 1999) (Fig. 1).

The “guard” hypothesis

One function of AVR proteins is likely to contribute to virulence of the pathogen by manipulation of certain (virulence) targets present in the host. The ongoing battle between plants and pathogens could in turn have led to development of strategies by which R proteins act as “guards” to monitor the behavior of molecules that are targets of AVR proteins. Van der Biezen et al. (1998) have proposed that the function of AvrPto for *P. syringae* is to target Pto and suppress the non-specific defense pathway induced by this kinase. The AvrPto-Pto complex or AvrPto-activated Pto is recognized by Prf, which subsequently initiates defense responses. A variety of mutations has been identified that disrupt the avirulence function of AvrPto without affecting its virulence function (Chang et al., 2001). Moreover, these mutants failed to interact with Pto, which, in line with the “guard” hypothesis, implies that AvrPto interacts with virulence targets other than Pto.

The “guard” hypothesis could also explain why, in spite of the fact that extracellular perception of AVR9 is consistent with the predicted extracellular location of Cf-9, no direct interaction between AVR9 and Cf-9 has been detected (Luderer et al., 2001b). Moreover, an AVR9-specific high affinity-binding site (HABS) has been identified in plasma membranes of susceptible *Cf-0*- as well as resistant *Cf-9* tomato plants, implying the involvement of a third component in *Cf-9*-mediated perception of AVR9 (Kooman-Gersmann et al., 1996). One possibility could be that the HABS represents the virulence target of AVR9.

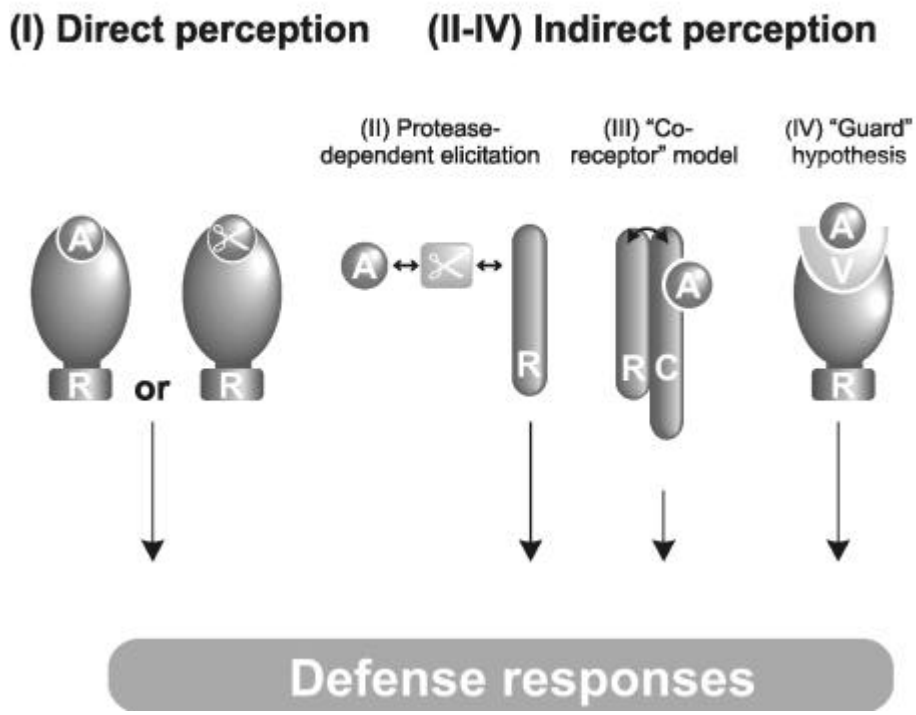


Fig. 1. Schematic representation of gene-for-gene interactions at the protein level. Four models have been proposed that describe either direct (I) or indirect (II-IV) perception of avirulence (AVR) proteins by plant resistance (R) proteins. (I) This classical model predicts a direct interaction between an AVR protein (A) and a matching R protein. Defense responses are induced independently or dependently, as illustrated by *scissors*, of the proteolytic activity of the AVR protein. (II) Binding of AVR protein to host-encoded proteins might involve the generation of protease-dependent elicitor peptide(s) or complex(es). The AVR protein, on the other hand, might also trigger *R*-gene mediated resistance by suppressing the generation of proteolytically processed negative regulators of defense responses. (III) The AVR protein binds to, at least, an additional component (C), which subsequently interacts with the R protein to trigger defense responses. The interaction between R protein and "co-receptor" might either be required for receptor activation after AVR binding or for recruitment of a functional receptor complex that mediates AVR recognition. (IV) According to the "guard" hypothesis, the R protein safeguards the virulence target (V) of the AVR protein. For further details, see text. (This figure has been modified from Bonas et al. 2002).

Cf-9 may "guard" this virulence target, sense its modification by AVR9 and trigger downstream defense responses leading to resistance (Van der Hoorn et al., 2002). Recent studies with tobacco cell suspensions revealed that *Cf-9*-mediated defense responses are attenuated at temperatures higher than 20°C and are completely suppressed at 33°C (De Jong et al., 2002). Interestingly, a correlation was found between the temperature-sensitivity of this response and the amount of AVR9-HABSs, suggesting that the HABS is unstable at elevated temperatures (De Jong et al., 2002).

The *Cf-4*-mediated defense responses are also temperature-sensitive, yet, opposed to AVR9, thus far no plant-derived AVR4-specific HABS has been detected (Chapter 3). As AVR4 exhibits chitin-binding activity, an interaction with chitin oligosaccharides might be required for AVR4 recognition by *Cf-4* plants. However, *Cf-4*-mediated defense responses are also triggered by AVR4 in the absence of chitin (De Jong et al., 2002), suggesting a perception mechanism independent of chitin. Thus, in order to be consistent with the "guard" hypothesis, AVR4 may function to bind to chitin, as well as to a host-encoded virulence target that is "guarded" by Cf-4.

Virulence targets can also include regulatory proteins that are involved in signal transduction pathways that activate basal or specific plant defense responses. Mutational analysis of *Arabidopsis* has identified several genes required for *NB-LRR* gene-mediated resistance toward the oomycete *P. parasitica* as well as the bacterium *P. syringae* (McDowell et al., 2000; Dangl and Jones, 2001). One of these genes, RIN4, has been proposed to be a target of the virulence activities of two elicitor proteins of *P. syringae*, AvrRpm1 and AvrB (Mackey et al., 2002). In the absence of RIN4, plants exhibit enhanced resistance toward *P. syringae* and *P. parasitica,* indicating that RIN4 negatively regulates the basal defense-signaling pathway. It has been suggested that interaction with and/or phosphorylation of RIN4 by AVR proteins enhances the activity of RIN4 as a negative regulator of basal plant defense. Thus, in the absence of RPM1, the AVR proteins function as virulence factors by manipulating RIN4 and suppressing basal defense mechanisms, whereas in resistant plants manipulation of RIN4 by AVR proteins is sensed by RPM1, which subsequently mounts a HR (Mackey et al., 2002).

The *RPP4* and *RPP5* resistance genes of *Arabidopsis thaliana* confer resistance to *P. parasitica.* The encoding proteins both interact with AtRSH1, a predicted cytoplasmic molecule with significant homology to bacterial RelA and SpoT protein (Van der Biezen et al., 2000). These RelA/SpoT proteins function as rapidly activated transcription cofactors in bacteria. AtRSH1 is proposed to mediate transcriptional activation of stress- and defense-related genes and compounds (Van der Biezen et al., 2000). In line with the "guard" hypothesis, pathogen-derived AVR proteins might interfere with the function of AtRSH1, and

RPP4 and RPP5 might have evolved to specifically recognize this physical association and subsequently activate defense responses (Van der Biezen et al., 2000).

The elicitin proteins of *Phytophthora* spp. behave like sterol carrier proteins. They can bind and pick up sterols from plasma membranes (Mikes et al., 1998). The ability of elicitins to load and transfer sterols correlates with their HR-inducing elicitor activity (Osman et al., 2001). Moreover, mutations that affect the affinity of different elicitins for sterol also seemed to affect the affinity for the HABS identified on plasmamembranes of tobacco cells (Osman et al., 2001). A model has been proposed in which binding of elicitins to the HABS requires the formation of a sterol-elicitin complex (Osman et al., 2001). Thus the HABS, which is composed of two plasmamembrane *N*-glycoproteins of 50 kDa and 162 kDa (Bourque et al., 1999), most likely evolved to recognize the sterol-elicitin complex. This heterodimeric HABS is also detected in plasma membranes of *Arabidopsis*, which is non-responsive toward elicitins (Bourque et al., 1999), indicating that additional components are required to trigger defense responses in Arabidopsis. One possibility could be that a functional, yet unidentified, R protein is present in tobacco, but not in Arabidopsis, which "guards" the interaction of the sterol-elicitin complex with the heterodimeric HABS and thereby confers disease resistance.

DEVELOPMENT OF TRANSGENIC PLANTS THAT DISPLAY BROAD RESISTANCE AGAINST PATHOGENS

Genetic analysis of plant disease resistance has demonstrated that single *R* gene products control resistance by mediating specific recognition, either directly or indirectly, of complementary AVR proteins produced by pathogens. Breeders have often used *R* genes to introduce resistance in their crops. However, the introgressed *R* genes, with only a few exceptions, have been shown to lack durability in the field (Stuiver and Custers, 2001). Early evidence arose from studies on the rust fungi *P. graminis* f. sp. *tritici* and *M. lini* that suggested a possible relationship between the durability of *R* genes and pathogen variation; *Avr* genes with low mutation rates corresponded to more durable resistance (Flor, 1958). Flor (1958) proposed that easily mutated *Avr* genes in pathogen populations are likely less critical to pathogen fitness than those that mutate rarely. Thus, if loss of avirulence function is associated with a reduced virulence of the pathogen, the complementary *R* gene is durable. Therefore, in order to confer durable resistance in crop plants, there is a great interest for cloning *R* genes, such as *Cf-ECP2* (Laugé et al., 1998b), which target *Avr* genes that are important for virulence.

De Wit (1992) proposed a strategy, which has been referred to as the two-component sensor system, to apply *Avr* genes in molecular resistance breeding. Thereby, an *Avr* gene is transferred together with its complementary *R* gene to a given crop plant. To specifically trigger defense responses upon pathogen challenge, one of the two components is placed under the control of a pathogen-inducible plant promoter, whereas the other is constitutively expressed. Activation of the gene cassette will lead to localized HR that will prevent further spread of the invading pathogen. The effectiveness of this HR-mediated resistance, however, relies on the ability of the pathogen to induce the promoter, as well as on the timing of, and sensitivity toward, the HR response. Yet, one of the major advantages of this strategy is to confer broad-spectrum disease resistance independent of AVR recognition and thus irrespective of the ability of pathogens to overcome *R* gene-mediated resistance. This strategy has been used to create transgenic plants that show broad-spectrum and high-level fungal control (Stuiver and Custers, 2001). As this strategy uses the endogenous defense components of the plant to engineer resistance, knowledge is required about how well downstream signaling components, including host-encoded virulence targets, are functionally conserved amongst plant species. For example, *R* genes frequently fail to function when transferred between plant species, especially when the species are not closely related. This suggests that the possibility to transfer resistance to commercially relevant crops by genetic engineering is often limited.

Transgenic tobacco plants expressing the elicitor protein cryptogein under the control of a pathogen-inducible promoter develop a HR in a normally compatible interaction with *P. parasitica* var *nicotiana* (Keller et al., 1999). These transgenic plants also display enhanced resistance to fungal pathogens that are unrelated to *Phytophthora* species (Keller et al., 1999). Furthermore, the oomycete *inf1* and fungal *Avr9* genes confer avirulence to PVX on tobacco and *Cf-9* tomato, respectively (Kamoun et al., 1999a). These results demonstrate that *Avr* genes can induce resistance to unrelated pathogens by the induction of a HR.

CONCLUSIONS AND FUTURE DIRECTIONS

To date, fifteen *Avr* genes of fungal origin have been cloned and demonstrated to govern either host genotype- or species-specificity. For some of these *Avr* genes sequence- or structural homology was found to known gene (products), thereby suggesting a putative intrinsic function for the pathogen. In the case of AVR9 and AVR-Pita, which exhibit homology to a carboxypeptidase inhibitor and a metalloprotease, respectively, no biochemical evidence is yet available confirming these putative functions. AVR4, on the other hand, exhibits structural homology to invertebrate chitin-binding domain proteins, binds

to chitin *in vitro*, and protects *T. viride*, and possibly *C. fulvum,* against cell wall disassembly by plant chitinases. In spite of the proposed contribution of several *Avr* genes to virulence of the fungus, a virulence role has only been assigned for the proteins encoded by *Ecp1, Ecp2* and *NIP1*. It is conceivable that *Avr* genes exhibit a role in virulence that is either difficult to quantify or is functionally compensated for by other effector proteins. One interesting field for future research would therefore be to create near-isogenic strains by gene-replacement or gene-silencing that differ only at the *Avr* loci, allowing subsequent detailed comparison on susceptible hosts to assess their contribution to virulence.

Recognition of AVR proteins is mediated by structurally different classes of R proteins. Detailed analysis of gene-for-gene pairs has provided further insight in how and where AVR proteins are recognized by resistant plant genotypes. Mutations in motifs that target AVR or R proteins to specific cellular compartments usually abolish AVR recognition by the corresponding R protein, suggesting that components required for triggering of defense responses are part of the same complex. This implies that recognition of extracytoplasmic proteins would occur at the extracellular side of plant plasmamembranes. Indeed, export of the CLV3 protein to the extracellular space of tomato is required for its interaction with and activation of the membrane-bound CLV1/CLV2 receptor complex. Yet, in the case of the extracellular AVR and ECP proteins of *C. fulvum*, for which the location allows an interaction with the extracellular LRRs of the Cf proteins, thus far no such interaction has been demonstrated. Moreover, evidence for direct interaction between AVR and R proteins is very limited and has only been demonstrated between AvrPto and Pto, and between AVR-Pita and Pi-ta.

For most other gene-for-gene pairs, models have been proposed that describe an indirect rather than a direct perception of AVR proteins by R proteins. Consistent with these models is the involvement of, at least, a third component in the perception complex. Such a component might represent a putative virulence target (like the AVR9-HABS) encoded by the host, which is guarded by the R protein. AVR proteins, however, might have more than one virulence target. This has been proposed for AvrPto, as mutants have been identified that retained their virulence function while their interaction with Pto was abolished, as well as for AVR4, for which binding to chitin is not required for induction of *Cf-4*-mediated resistance. Thus, in order to elucidate the intrinsic function of AVR proteins, it is important to identify their putative virulence targets. Indirect perception, on the other hand, might also involve co-receptors (like protein kinases) that form a complex with R proteins. Ligand-induced conformational changes or phosphorylation of receptors could facilitate transmission from the extracytoplasmic to the cytoplasmic domains and subsequently trigger signal transduction. Moreover, indirect perception could also involve protease-dependent

elicitation of defense responses, which has been proposed for AVR-Pita, which encodes a putative metalloprotease, as well as for AVR2, which requires a cysteine endoprotease Rcr3 to trigger *Cf-2*-mediated defense responses. Ongoing research will identify additional players that are involved in the current models of AVR perception by resistant plants.

Several *R* genes of the NB-LRR family have been cloned that confer resistance toward *M. lini* (*L6, M, N,* and *P*), *P. parasitica* (*RPP1, 4, 5, 8, 10, 13,* and *14*), *B. lactucae* (*Dm3*), *V. dahliae* (*Ve1* and *Ve2*), *F. oxysporum* (*I2*), and *P. infestans* (*R1*). Most of these fungal and oomycetous pathogens, however, are not very amenable to molecular manipulation, which hampers cloning of the matching *Avr* genes. Although the NB-LRR proteins lack any obvious subcellular targeting signatures, for at least one NB-LRR protein, RPM1, which recognizes intracellular targeted AvrRpm1 and AvrB, association with the plasmamembrane as a result of myristylation has been demonstrated (Boyes et al., 1998). The putative cytoplasmic localization of the NB-LRR proteins suggests intracellular perception of the *Avr* gene products. Most fungal pathogens that are recognized by these NB-LRR proteins, however, produce haustoria and grow extracellularly. This implies that these pathogens might use specialized mechanisms for delivery of AVR proteins into plant cells. Whether such mechanisms show parallels with the bacterial *Hrp*-mediated type III secretion system (Bonas and Van den Ackerveken, 1997) needs to be elucidated.

The maintenance of *Avr* genes in pathogen populations suggests that the primary function of these genes is to contribute to virulence of the pathogen. A focus for future research should involve breeding strategies aimed at plant resistance directed against essential effector proteins of pathogens, which will lead to development of crop species that harbor durable resistance. However, to achieve such durability, more insight is required into the molecular mechanisms underlying the adaptation of pathogens to *R* gene-mediated resistance.

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Outline of the thesis

Cladosporium fulvum is a biotrophic fungus that causes leaf mould of tomato. During colonization of the extracellular space of tomato leaves, various proteins are secreted, amongst which are the small and stable, cysteine-rich avirulence (AVR) proteins. These AVR proteins elicit defense responses, including a hypersensitive response (HR), in plants expressing the matching *Cf* resistance genes. The *Cf-4* and *Cf-9* resistance genes belong to a large family of homologues of *C. fulvum* resistance gene *Cf-9* (*Hcr9*s) that confer resistance to *C. fulvum* through (in)direct recognition of the *Avr4* and *Avr9* gene products, respectively.

At the *Cf-4* locus, homologue *Hcr9-4E* was found to confer resistance, independent of the *Cf-4* gene, towards strains of *C. fulvum* carrying a novel avirulence determinant designated *Avr4E.* To examine whether *Hcr9-4E* is as effective as *Cf-4* in conferring resistance, apoplastic fluid isolated from *C. fulvum*-infected tomato plants that express *Hcr9-4E* was assayed for the presence of ECP2, a virulence protein that is secreted by all strains of *C. fulvum* during growth in tomato leaves (Chapter 2). It appeared that *Hcr9-4E* is indeed a functional resistance gene that recognizes a yet unknown avirulence determinant, designated AVR4E. Therefore, we set out to purify the AVR4E protein from apoplastic fluid of *C. fulvum*-infected tomato leaves and to clone and characterize the *Avr4E* gene (Chapter 2). Furthermore, we studied the molecular mechanism underlying the evasion of *Cf*-mediated resistance. We found that strains of *C. fulvum* evade *Hcr9-4E*-mediated resistance either by lack of *Avr4E* expression or by production of a stable, elicitor-inactive AVR4E mutant protein that carries two amino acid substitutions, Phe^{62}-to-Leu and Met^{73}-to-Thr.

The *Cf* genes confer resistance to strains of *C. fulvum* through recognition of different avirulence determinants by a mechanism that still needs to be elucidated. There are indications that Cf-9 plays an indirect role in the perception of AVR9 and that at least a third component is involved in the perception of AVR9 by plants carrying the *Cf-9* gene. To examine whether a similar situation holds for perception of AVR4, we performed binding studies with ^{125}I-AVR4 on microsomal membranes fractions (MFs) of MM-Cf0 (susceptible) and MM-Cf4 (resistant) tomato plants and non-host plant species (Chapter 3). We identified an AVR4 high-affinity binding site (HABS) in MFs that appeared to originate from fungi present on partially infected tomato leaves rather than from the tomato plant itself. Detailed analyses showed that this fungus-derived AVR4-specific HABS is proteinase K- and heat-resistant. In addition, affinity crosslinking experiments demonstrated that AVR4 specifically interacts with a component of approximately 75 kDa that is of fungal origin. When binding was performed on MFs isolated from plants that were grown under contained conditions, no

AVR4-specific HABS could be detected. This implies that the mechanism of perception of AVR4 by plants carrying *Cf-4* is different from that described for several other fungus-derived elicitor proteins, including AVR9, for which a HABS has been found in MFs of several plant species.

Several natural strains of *C. fulvum* have been identified to evade *Cf-4*-mediated resistance by production of AVR4 mutant proteins carrying a Cys-to-Tyr substitution. As demonstrated by recombinant potato virus-X (PVX)-mediated expression, it appeared that some of these AVR4 mutant proteins still exhibit residual elicitor activity, while others are no longer recognized by plants carrying the *Cf-4* gene. To determine which of the disulfide bonds present in AVR4 are essential for conformational stability and elicitor activity of AVR4, all eight cysteine residues were individually replaced by alanine and, subsequently the mutant proteins were expressed in PVX and recombinant PVX constructs were inoculated onto MM-Cf4 plants to assay for necrosis-inducing activity (NIA) (Chapter 4). Partial chemical reduction of the disulfide bonds was also performed, followed by mass spectrometry, which resulted in confirmation of disulfide bonds identified by PVX experiments and in assignment of the remaining disulfide bonds in AVR4. The disulfide bond pattern and the spacing of the cysteine residues were subsequently used to carry out a motif search, which revealed that AVR4 contains an invertebrate chitin-binding domain (inv ChBD). It appeared that three disulfide bonds in AVR4, which are conserved amongst members of the inv ChBD family, are required for conformational stability of AVR4. Moreover, strains of *C. fulvum* were found to evade *Cf-4*-mediated resistance by secretion of unstable disulfide bond mutants of AVR4 that retained their ability to bind to chitin.

In Chapter 5, we analyzed whether domains responsible for NIA and chitin-binding ability within the AVR4 protein can be distinguished. An antibody-affinity analysis of overlapping, synthetic peptides (PEPSCAN) was performed to identify antigenic domains in AVR4 containing putatively solvent-exposed residues that might condition NIA of AVR4. Alanine substitutions were subsequently introduced for residues that are present in the major antigenic domain of AVR4. Moreover, all aromatic residues and two additional residues that are present in conserved domains of hypothetical chitin-binding structures of AVR4 were also replaced independently by alanine. The results described in Chapter 5 suggest that multiple, rather than single, amino acid substitutions in AVR4 are required to obtain a reduction in the NIA and the chitin-binding ability of AVR4.

In the summarizing discussion (Chapter 6), the different resistance specificities conferred by *Hcr9*s are discussed. Moreover, we discuss a potential phylogenetic relationship between the different *C. fulvum* isolates, we speculate about the role of AVR4

and the *Cf-4* gene in co-evolution between *C. fulvum* and tomato and we elaborate on the role of other AVRs in virulence and avirulence of *C. fulvum*.

Chapter 2

Cladosporium fulvum evades *Hcr9-4E*- mediated resistance by abolishing *Avr4E* expression or by modifying the AVR4E elicitor protein

Part of this chapter has previously been published (Takken et al. 1999). A modified version of part of this chapter will be submitted for publication by Nienke Westerink, Bas F. Brandwagt, Pierre J. G. M. de Wit and Matthieu H. A. J. Joosten.

Cladosporium fulvum evades *Hcr9-4E*-mediated resistance by abolishing *Avr4E* expression or by modifying the AVR4E elicitor protein

Abstract

Introgression of resistance trait *Cf-4* from wild tomato species into tomato cultivar MoneyMaker has resulted in near-isogenic line MM-Cf4 that confers resistance to *Cladosporium fulvum* through recognition of AVR4. Within the *Cf-4* introgressed region, five homologues of *Cladosporium* resistance gene *Cf-9* (*Hcr9*s) are present. While *Hcr9-4D* represents the functional *Cf-4* resistance gene mediating recognition of AVR4, *Hcr9-4E* confers resistance towards *C. fulvum* upon recognition of a novel avirulence determinant, designated AVR4E. Here, we demonstrate that *Hcr9-4E* is a functional resistance gene and we cloned the *Avr4E* gene, which encodes a cysteine-rich protein of 101 amino acids that is secreted into the apoplastic space during growth of *C. fulvum* in tomato leaves. Complementation studies showed that AVR4E confers avirulence to strains of *C. fulvum* that are normally virulent on *Hcr9-4E*-transgenic plants, indicating that AVR4E is a genuine avirulence determinant. It appeared that strains of *C. fulvum* evade *Hcr9-4E*-mediated resistance either by lack of *Avr4E* gene expression or by production of a stable AVR4E mutant protein that carries two amino acid substitutions, Phe^{62}-to-Leu and Met^{73}-to-Thr. Moreover, we demonstrated by site-directed mutagenesis that single amino acid substitution Phe^{62}Leu reduced the elicitor activity of AVR4E, indicating that this substitution in AVR4E might be sufficient to evade *Hcr9-4E*-mediated resistance.

Introduction

The biotrophic fungal pathogen *Cladosporium fulvum* causes leaf mould on its only host tomato. During colonization, *C. fulvum* secretes several proteins into the apoplastic space of tomato leaves, among which are race-specific elicitor proteins encoded by avirulence (*Avr*) genes. These AVR proteins induce defense-related responses, including a hypersensitive response (HR), in tomato plants that carry the corresponding *Cf* resistance (*R*) genes (Joosten and De Wit, 1999). The *Cf* genes encode proteins with a predicted signal peptide, an extracellular leucine-rich repeat (LRR) region, a transmembrane domain and a short cytoplasmic tail. The highest degree of amino acid variability is found in the N-terminal,

solvent-exposed residues of the LRR parallel â-sheet structure, which is implicated to determine resistance specificity.

The *Cf* genes are organized in clusters of homologues, which have been designated *Hcr2*s and *Hcr9*s for homologues of *Cladosporium* resistance gene *Cf-2* and *Cf-9*, respectively. Whilst the *Cf-2* and *Cf-5* loci map at identical locations on the short arm of chromosome 6 of tomato (Balint-Kurti et al., 1994), the *Cf-4* and *Cf-9* loci map at the short arm of chromosome 1 of tomato (Jones et al., 1993). At the *Cf-2* locus, the nearly identical *Cf-2.1* and *Cf-2.2* genes confer resistance towards strains of *C. fulvum* carrying the matching *Avr2* gene, whereas a third homologue *(Hcr2-2A)* is nonfunctional (Dixon et al., 1996). Four other *Hcr2* homologues are found at the *Cf-5* locus, of which *Hcr2-5C* is the functional *Cf-5* gene that was thought to mediate recognition of the not yet characterized *Avr5* gene product (Dixon et al., 1998). The *Cf-4* and *Cf-9* gene clusters, which originate from wild species of the genus *Lycopersicon*, have been introgressed in tomato cultivar MoneyMaker (MM), resulting in near-isogenic lines MM-Cf4 and MM-Cf9, respectively (Stevens and Rick, 1988; Boukema, 1981). Each locus comprises five *Hcr9*s, of which *Hcr9-4D* and *Hcr9-9C* are the functional *Cf-4* and *Cf-9* genes, respectively (Parniske et al., 1997; Thomas et al., 1997 and 1998), as these homologues confer resistance to *C. fulvum* through recognition of AVR4 and AVR9, respectively (Van Kan et al., 1991; Joosten et al., 1994).

Within the *Hcr9* gene family, resistance specificities conferred by genes other than *Cf-4* and *Cf-9* have been identified. At the *Cf-9* locus, *Hcr9-9B* and *Hcr9-9E* were found to provide weak resistance towards *C. fulvum* infection, in particular towards *C. fulvum* races 4, 5, and 5.9, suggesting that these homologues mediate recognition of avirulence determinants other than AVR4 and AVR9 (Parniske et al., 1997; Panter et al., 2002). Moreover, this weak resistance was only observed in adult plants and is clearly different from *Cf-9*-mediated resistance induced by recognition of AVR9, as some disease symptoms are still present on these plants (Parniske et al., 1997; Laugé et al., 1998a; Panter et al., 2002).

Takken et al. (1998) identified two *Cf-4* mutant plants carrying a *Ds* transposon insertion in the open reading frame (ORF) of *Hcr9-4E*. As this insertion also affected the function of *Cf-4*, these mutant *Cf-4* plants were found to be susceptible towards strains of *C. fulvum* that produce the AVR4 elicitor protein (race 5) (Takken et al., 1998). Moreover, a *Ds* transposon insertion within homologue *Hcr9-4D* or a deletion of the ORF of *Hcr9-4D* resulted in mutants that still conferred resistance towards *C. fulvum* (Takken et al., 1999), which suggests that *Hcr9-4E* also exhibits the ability to mediate disease resistance. Indeed, by performing complementation experiments, it appeared that homologue *Hcr9-4E* conferred resistance towards *C. fulvum*, independent of *Hcr9-4D,* through recognition of a novel AVR

determinant, which has been designated AVR4E. Opposed to *Hcr9-4E,* homologues *Hcr9-4A, Hcr9-4B* and *Hcr9-4C* are non-functional in conferring resistance towards *C. fulvum* race 5, as these homologues are present in five *Cf-4*/*Cf-9* recombinant plants that are susceptible to this race (Parniske et al., 1997; Thomas et al., 1997).

Here, we demonstrate that *Hcr9-4E* confers the same level of resistance towards *C. fulvum* as *Hcr9-4D.* We also purified AVR4E from apoplastic washing fluids of *C. fulvum*-infected tomato leaves and subsequently cloned the encoding *Avr4E* gene. *Avr4E* encodes a cysteine-rich protein, of which the mature form contains 101 amino acids, which is secreted into the apoplastic space of tomato leaves. Complementation studies showed that AVR4E confers avirulence to strains of *C. fulvum* that are normally virulent on *Hcr9-4E*-transgenic plants, demonstrating that AVR4E is a genuine avirulence determinant. It appeared that *C. fulvum* circumvents *Hcr9-4E*-mediated resistance either by lack of *Avr4E* gene expression or by production of a stable AVR4E mutant protein that carries two amino acid substitutions Phe^{62}Leu and Met^{73}Thr. Moreover, we showed by site-directed mutagenesis that single amino acid substitution Phe^{62}Leu reduced the elicitor activity of AVR4E, indicating that this substitution in AVR4E might be sufficient to evade *Hcr9-4E*-mediated resistance.

Results

Transgenic tomato plants expressing *Hcr9-4E* are resistant to *C. fulvum* race 5

Cf-4 mutant plants have been identified that lack a functional *Cf-4* resistance gene, as a result of a deletion of *Cf-4* or a transposon insertion in the open reading frame (ORF) of *Cf-4* (Takken et al., 1999). These *Cf-4* mutant plants still confer resistance towards *C. fulvum* race 5, a strain that produces the AVR4 elicitor protein. To examine whether this resistance phenotype was mediated by homologue *Hcr9-4E*, adult *Hcr9-4E*-transgenic plants were inoculated with *C. fulvum* race 5. Although some fungal growth was observed on primary leaves of both 3-week-old *Hcr9-4D*- and *Hcr9-4E*-transgenic plants (data not shown), adult *Hcr9-4E*-transgenics appeared to be as resistant as *Hcr9-4D* transgenic or MM-Cf4 plants that carry the complete *Cf-4* cluster (Fig. 1). Transgenic plants expressing *Hcr9-4C,* however, are susceptible to *C. fulvum* race 5 (Fig. 1), which is consistent with previously obtained results (Thomas et al., 1997).

The level of resistance conferred by *Hcr9-4D* and *Hcr9-4E* is the same

To further analyze whether *Hcr9-4E* confers the same level of resistance towards *C. fulvum* as *Hcr9-4D, Ecp2* expression analysis was performed on (transgenic) tomato plants that

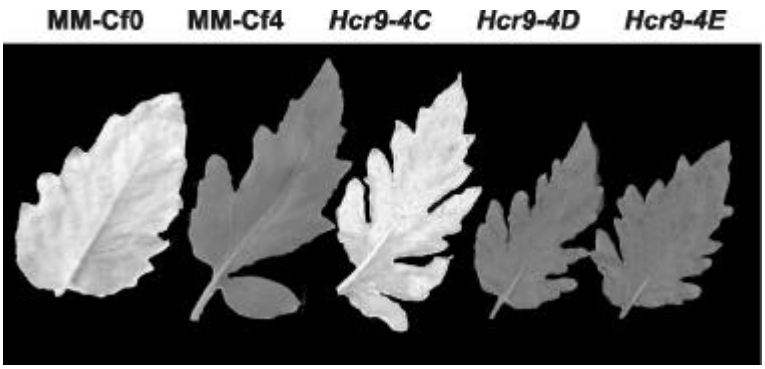

Fig. 1. Disease symptoms on six-week-old (transgenic) tomato plants inoculated with *C. fulvum* race 5. Six-week-old MM-Cf0 and MM-Cf4 plants and transgenic tomato plants expressing *Hcr9-4C, Hcr9-4D,* or *Hrc9-4E* were inoculated with *C. fulvum* race 5. Disease symptoms present at the lower side of the leaflets were scored three weeks post inoculation. Note that only MM-Cf0 and *Hcr9-4C*-transgenic plants are diseased, while MM-Cf4 and transgenic plants expressing *Hcr9-4D* or *Hcr9-4E* are free of symptoms.

were inoculated with *C. fulvum* race 5. During a compatible interaction on tomato plants, strains of *C. fulvum* were found to secrete ECP2, an essential virulence protein (Laugé et al., 1997), which was therefore used as a marker for fungal growth (Wubben et al., 1994). Two-to-three-weeks after inoculation, apoplastic fluid (AF) was isolated from leaves of 3- and 6-week-old (transgenic) tomato plants and subjected to Western blot analysis. Some ECP2 protein was detected in AF obtained from 3-week-old, *C. fulvum*-infected plants that express *Hcr9-4D* or *Hcr9-4E* (Fig. 2A). However, a significant higher level of ECP2 protein was observed in AF isolated from 3-week-old MM-Cf0 plants or *Hcr9-4C*-transgenic plants (Fig. 2). Opposed to the 3-week-old transgenic plants, no ECP2 protein could be detected in AF obtained from 6-week-old, *C. fulvum*-infected *Hcr9-4D*- or *Hcr9-4E*-transgenic plants (Fig. 2B). These results were confirmed by cytological analysis on *C. fulvum*-inoculated tomato leaves, whereby on 3-week-old *Hcr9-4D*- and *Hcr9-4E*-transgenic plants occasionally some mycelium was observed (data not shown). Possibly, 3-week-old seedlings exhibit a lower transgene expression level than adult plants, thereby resulting in emergence of some fungal growth. Nevertheless, these data demonstrate that *Hcr9-4E* confers the same level of resistance towards *C. fulvum* race 5 as *Hcr9-4D*.

Hcr9-4E recognizes a novel AVR determinant that is designated AVR4E

When *Hcr9-4E*-transgenic plants were inoculated with potato virus X (PVX)::*Avr4*, no necrotic symptoms developed (Thomas et al., 1997). Moreover, *Hcr9-4E* transgenics as well as plants that lack a functional *Cf-4*, but carry a functional *Hcr9-4E*, exhibited no seedling lethality when crossed to transgenic plants expressing *Avr4* (Thomas et al., 1997; Takken et al., 1999). This implies that *Hcr9-4E* confers resistance towards *C. fulvum* race 5 through recognition of an avirulence determinant that is different from AVR4, which we designated AVR4E. To show that, in addition to AVR4, AVR4E is secreted by *C. fulvum* race 5, AF was isolated from *C. fulvum* race 5-infected MM-Cf0 leaves. When this AF was injected into leaves of *Hcr9-4D* transgenics and MM-Cf4 plants necrotic lesions developed, whereas a more chlorotic response developed in leaves of *Hcr9-4E*-transgenic plants (Fig. 3). This AF did not induce necrosis or chlorosis upon injection into leaves of *Hcr9-4C* transgenics or MM-Cf0 plants (Fig. 3). Moreover, necrotic lesions were induced when AF was injected into leaves of transgenic *Nicotiana tabacum* plants expressing *Hcr9-4D* or *Hcr9-4E*, especially when these genes were under control of the 35S promoter of cauliflower mosaic virus (data not shown). Together, these data demonstrate that novel avirulence determinant AVR4E can be detected in AF through its necrosis-inducing activity (NIA) on plants that carry *Hcr9-4E*.

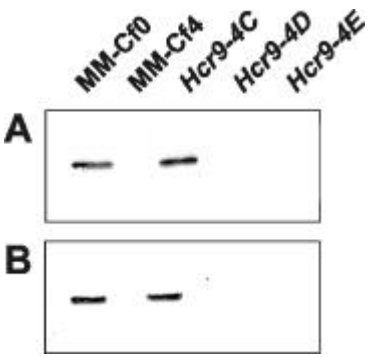

Fig. 2. Immunodetection of the ECP2 protein in the apoplastic space of *C. fulvum*-infected (transgenic) tomato leaves. A, Three-week-old and **B,** six-week-old MM-Cf0 and MM-Cf4 plants, and transgenic tomato plants expressing *Hcr9-4C*, *Hcr9-4D*, or *Hcr9-4E*, under control of their native promoter, were inoculated with *C. fulvum* race 5. Apoplastic-washing fluid (AF) was isolated from these *C. fulvum*-infected leaves, three weeks post inoculation. Proteins present in 5 µl of AF were separated by SDS-PAGE and Western blot analysis was performed, using antibodies raised against ECP2, a protein that is used as a marker for successful infection. Note that ECP2 protein is only detected in AF of susceptible MM-Cf0 and *Hcr9-4C*-transgenic plants.

Isolation of the gene encoding avirulence determinant AVR4E

To isolate AVR4E, proteins present in AF of *C. fulvum* race 5-infected tomato leaves were fractionated by gel filtration and the fractions were assayed for NIA by injection into leaves of *Hcr9-4E* transgenic plants. Proteins present in fractions that exhibited NIA were subjected to

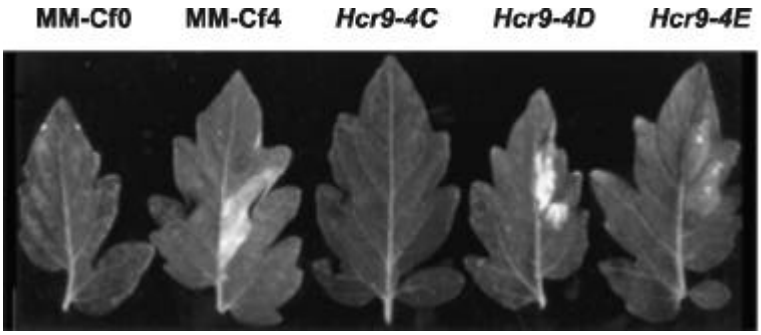

Fig. 3. Necrosis-inducing activity of apoplastic fluid from a compatible *C. fulvum*-MM-Cf0 interaction after injection into (transgenic) tomato leaves (Takken et al., 1999; Fig. 4). Apoplastic fluid (AF) obtained from leaves of *C. fulvum* race 5-infected MM-Cf0 was injected into leaves of MM-Cf0 and MM-Cf4 plants and transgenic tomato plants expressing *Hcr9-4C*, *Hcr9-4D* or *Hcr9-4E*. Note that only resistant plants show necrosis or chlorosis (see also Figs. 1 and 2).

anion exchange chromatography and fractions that exhibited biological activity in *Hcr9-4E* transgenics were subsequently purified by reverse-phase chromatography (Fig. 4). Following these purification steps, we identified a protein that exhibited specific NIA in *Hcr9-4E*-transgenic plants (Fig. 4; fraction III).

From this protein, which we tentatively designated AVR4E, an N-terminal and an internal amino acid sequence was determined. To isolate the gene encoding AVR4E, anchored PCR on genomic DNA of *C. fulvum* race 5 was performed, using degenerate oligonucleotides that were designed based on the determined amino acid sequences. In this way, we obtained the 5'-end of *Avr4E*, which was subsequently used to screen a cDNA library obtained from leaves of *C. fulvum* race 5-infected MM-Cf0 plants, which resulted in isolation of the full-length cDNA of *Avr4E* (Fig. 5). To isolate the genomic sequence of *Avr4E,* we screened a genomic library from *C. fulvum* race 4, as a genomic library of *C. fulvum* race 5 was not available at the time. This screening resulted in the identification of the promoter and terminator region of the *Avr4E* sequence of race 4, which subsequently allowed isolation of the genomic *Avr4E* sequence of race 5 by PCR.

Comparison of the *Avr4E* cDNA sequence with the genomic *Avr4E* sequence of *C. fulvum* race 5 revealed that the *Avr4E* gene does not contain introns (Fig. 5). Moreover, Southern blot analysis of *C. fulvum* race 4 and race 5 showed that *Avr4E* is present as a single-copy gene in the genome of these strains (data not shown). The promoter region, which is nearly identical in race 4 and race 5, contains the consensus sequences (TA)GATA

and CAAT (Fig. 5). The (TA)GATA sequences are involved in regulation of *Avr9* gene expression under nitrogen-limiting conditions *in vitro* and *in planta* (Snoeijers et al., 1999; Pérez-García et al., 2001), while the CAAT sequences play a role in conidiophore development (Adrianopoulos and Timberlake, 1994).

AVR4E behaves as a genuine avirulence determinant

To determine whether the isolated protein represents the product encoded by avirulence gene *Avr4E,* two strains of *C. fulvum* virulent on *Hcr9-4E*-transgenic plants, i.e. strain South America (SA) and race 4, were transformed with a genomic *Avr4E* clone of race 5. For strain SA, virulence on *Hcr9-4E* transgenics is not associated with virulence on *Hcr9-4D* transgenics, indicating that strain SA behaves like a race 4E (Fig. 6A). Unlike strain SA, *C. fulvum* race 4 is virulent on *Hcr9-4D*-transgenic plants and MM-Cf4 plants that carry the complete *Cf-4* locus, indicating that race 4 does not produce a functional AVR4 elicitor protein (Fig. 6C) (Joosten et al., 1994 and 1997).

Insertion of the genomic *Avr4E* sequence of race 5 into the genome of strain SA and *C. fulvum* race 4 resulted in transformants that are avirulent on *Hcr9-4E* transgenics as well as on MM-Cf4 plants (Fig. 6B and 6D). To show that these transformants indeed secrete a functional AVR4E protein, AF was isolated from susceptible MM-Cf0 plants that were infected with either these transformants or the parent strains. Only AF obtained from transformant-infected tomato showed NIA when injected into leaves of *Hcr9-4E*-transgenic plants (data not shown). These results demonstrate that the isolated *Avr4E* gene indeed encodes the race-specific avirulence determinant AVR4E, which confers avirulence to strains that are normally virulent on plants that carry *Hcr9-4E*.

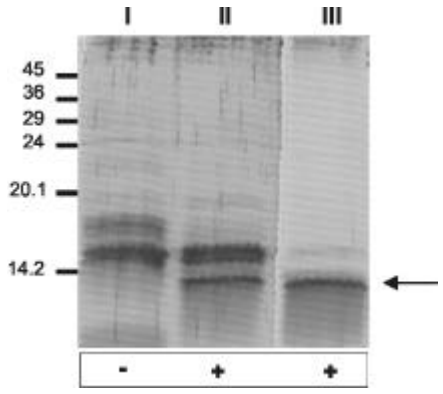

Fig. 4. Fractionation and isolation of the AVR4E protein from apoplastic fluid of *C. fulvum* race 5-infected MM-Cf0 plants. Proteins present in apoplastic-washing fluid (AF) of a compatible interaction between *C. fulvum* race 5 and MM-Cf0 were separated by gel filtration. The fractions that exhibited necrosis-inducing activity (NIA) when injected into leaves of *Hcr9-4E*-transgenic plants were subjected to anion exchange chromatography. Subsequent reverse-phase chromatography of the fractions showing NIA resulted in fractions (II and III) that still exhibited NIA in leaves of *Hcr9-4E* transgenics, as indicated (+). From the protein present in fraction III (indicated by an arrow), a partial amino acid sequence was obtained.

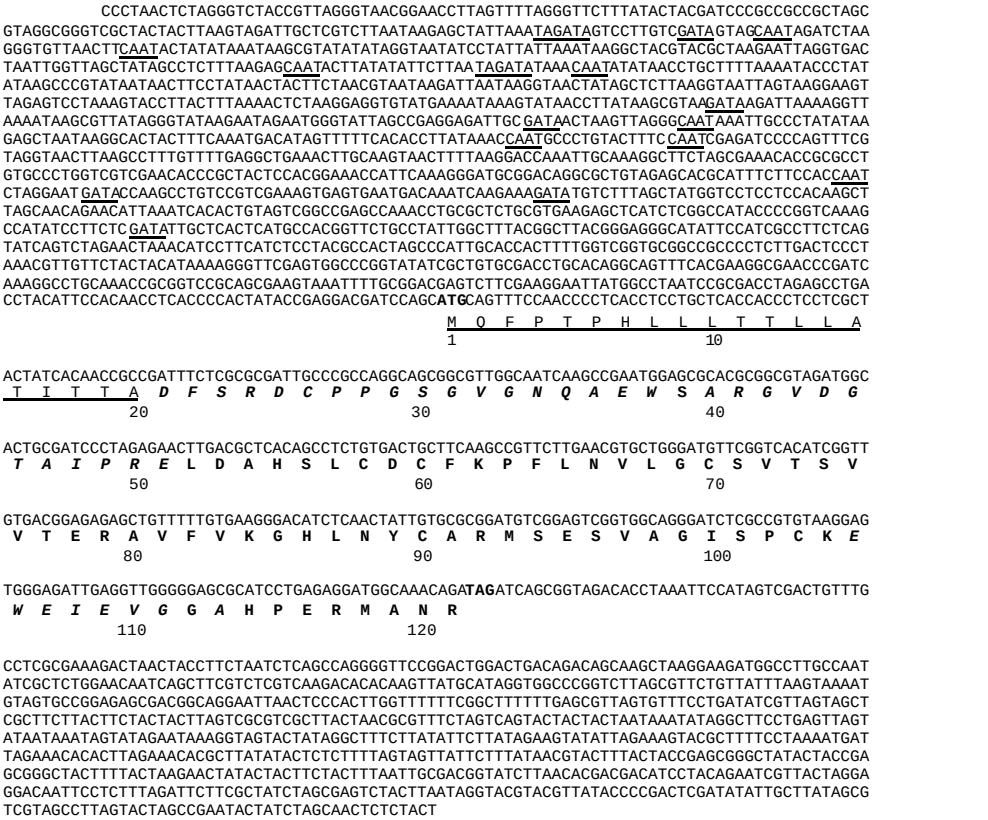

Fig. 5. DNA and protein sequence of the AVR4E elicitor protein. The genomic sequence of the *Avr4E* gene of *C. fulvum* is shown. The *Avr4E* gene encodes a pre-protein that contains a 20-amino acid N-terminal signal peptide (underlined) for extracellular targeting. The mature form of the AVR4E protein consists of 101 amino acids (bold), including six cysteines. The consensus sequences (TA)GATA and CAAT present in the promoter are underlined. The N-terminal- and internal amino acid sequences derived from the purified AVR4E protein are indicated (italics).

AVR4E is a small, secreted cysteine-rich protein

The *Avr4E* ORF encodes a predicted protein of 121 amino acids, which consists of six cysteine residues. The signal peptide cleavage site of AVR4E was predicted using the SignalP program (Nielsen et al., 1997), which identified a 20-amino acid N-terminal signal sequence for extracellular targeting (Fig. 5). The predicted cleavage site is consistent with the sequence information obtained from N-terminal sequencing of AVR4E purified from AF (Fig. 4), demonstrating that the N-terminus of AVR4E is not further processed by fungal- or

plant proteases in the apoplastic space of tomato. Assuming that C-terminal processing does not occur, the mature AVR4E protein consists of 101 amino acids. No significant sequence homology could yet be found between the *Avr4E* gene, or the AVR4E protein, and sequences present in the database.

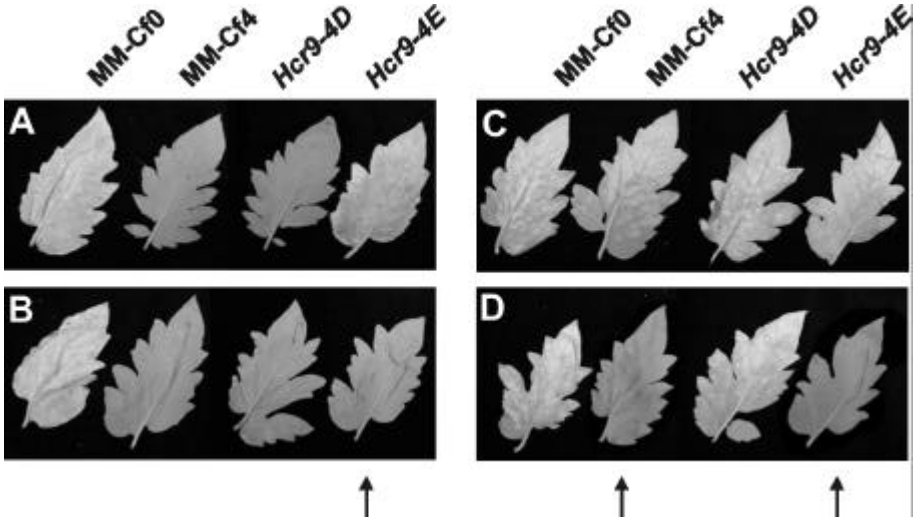

Fig. 6. AVR4E behaves as a genuine avirulence determinant on tomato plants carrying *Hcr9-4E.* Leaves of MM-Cf0 and MM-Cf4 plants, and transgenic plants expressing *Hcr9-4D* or *Hcr9-4E* were inoculated with strain SA (A) and race 4 (C), or with four independent transformants of strain SA (B) and race 4 (D), carrying the *Avr4E* genomic sequence of race 5. Photographs of the lower side of the leaflets were taken three weeks post inoculation. Note that no disease symptoms are present on leaves of *Hcr9-4E*-transgenic plants and MM-Cf4 plants that were inoculated with transformants that carry *Avr4E* (indicated by arrows in B and D).

C. fulvum employs two different mechanisms to evade AVR4E recognition

As demonstrated by *C. fulvum* race 4, strains of *C. fulvum* have developed the ability to evade both *Hcr9-4D*- and *Hcr9-4E*-mediated resistance (Fig. 6C). Previously, it has been demonstrated that, in most cases, strains circumvent *Hcr9-4D*-mediated resistance by production of AVR4 isoforms that carry different single amino acid substitutions (Joosten et al., 1994 and 1997).

To investigate whether loss of AVR4E recognition was also accomplished by production of mutated AVR4E isoforms, we PCR-amplified the *Avr4E* ORF from the genome of 25 strains of *C. fulvum* that were virulent and 10 strains that were avirulent on *Hcr9-4E*-

transgenic plants. Sequence analysis of these PCR products revealed that all avirulent strains carry an *Avr4E* sequence with an ORF identical to that of the *Avr4E* gene present in *C. fulvum* race 5 (Fig. 5). However, for 17 strains of *C. fulvum* virulent on *Hcr9-4E*-transgenic plants, among which are race 4 and race SA, a similar modification was detected in the ORF of the *Avr4E* gene. This modification involves a double point mutation, resulting in amino acid substitutions, Phe^{62}-to-Leu and Met^{73}-to-Thr (avr4E^{LT}). To analyze whether avr4E^{LT} still exhibits NIA, we employed the PVX-based expression system (Chapman et al., 1992). In spite of the fact that the native signal peptide of AVR2, AVR4 and AVR9 has been reported to mediate proper extracellular targeting of these proteins when expressed in tomato using PVX (Takken et al., 2000a), the endogenous signal peptide of AVR4E did not (data not shown). Therefore, PVX::*Avr4E* and PVX::*avr4E^{LT}* were obtained by cloning the sequence encoding mature AVR4E and avr4E^{LT} protein, respectively, downstream of the sequence encoding the PR-1a signal peptide of *Nicotiana tabacum* (Hammond-Kosack et al., 1995). Tomato plants were subsequently inoculated with the recombinant PVX vectors and subsequently examined for the development of systemic HR (Fig. 7). It appeared that *Hcr9-4E*-transgenic plants and MM-Cf4 plants inoculated with PVX::*Avr4E* developed a clear systemic HR, which eventually resulted in plant death. When *Hcr9-4E* transgenics were inoculated with PVX::*avr4E^{LT}*, systemic mosaic symptoms were detected that were caused by the PVX infection (Fig. 7). Thus, unlike AVR4E, avr4E^{LT} does not exhibit NIA, demonstrating that strains of *C. fulvum* evade *Hcr9-4E*-mediated resistance by modifying AVR4E in such way that plants carrying *Hcr9-4E* do no longer recognize this isoform.

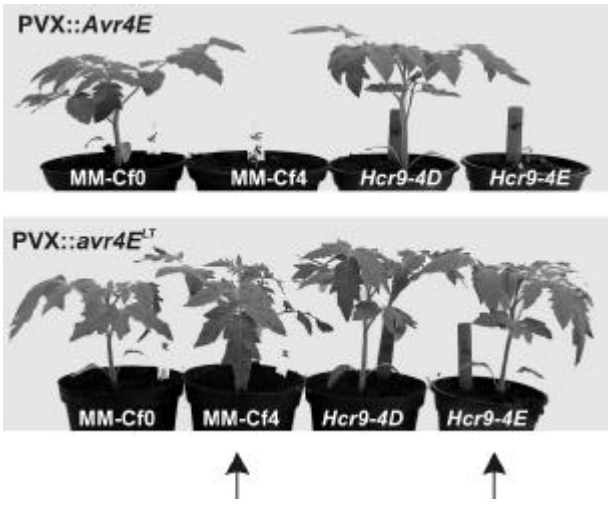

Fig. 7. PVX-mediated expression of *Avr4E* and *avr4E^{LT}* in(transgenic) tomato plants. Three-week-old MM-Cf0 and MM-Cf4 plants and transgenic plants expressing *Hcr9-4D* or *Hcr9-4E* were inoculated with PVX::*Avr4E* (upper panel) or PVX::*avr4E^{LT}* (lower panel). Plants were photographed 14 days post inoculation with PVX. Note that, opposed to PVX::*Avr4E*, PVX::*avr4E^{LT}* does not cause systemic necrosis in MM-Cf4 and *Hcr9-4E*-transgenic plants.

Interestingly, all other strains virulent on *Hcr9-4E*-transgenic plants (i.e. 8 out of 25) carry an *Avr4E* ORF that is identical to that of strains avirulent on *Hcr9-4E*-transgenic plants. To analyze whether these 8 virulent strains produced the AVR4E protein, AF was isolated from MM-Cf0 plants that were infected by these virulent strains and injected into leaves of *Hcr9-4E* transgenic plants. No NIA was observed (data not shown), indicating that these virulent strains do not produce a functional AVR4E elicitor protein. Moreover, transformation of these virulent strains with a genomic *Avr4E* clone of *C. fulvum* race 5 resulted in avirulence on *Hcr9-4E* transgenics, as confirmed by 8 independent transformants of two parent strains (data not shown).

To further investigate the mechanism conditioning virulence of these strains on plants carrying *Hcr9-4E*, the expression of *Avr4E* during growth on susceptible tomato plants was investigated. For strains of *C. fulvum* that are avirulent (race 5) as well as strains that are virulent on these plants and carry *avr4E*^{LT} (strain SA and race 4), *Avr4E* transcripts accumulated during growth on tomato (Group I and II; Fig. 8). However, for strains that are virulent on *Hcr9-4E*-transgenic plants and carry a wild-type *Avr4E* ORF, no *Avr4E* transcripts could be detected (Group III; Fig. 8). However, transformation of these strains with the genomic *Avr4E* clone of race 5 resulted in accumulation of *Avr4E* transcripts *in planta* (Group IV; Fig. 8). Altogether, these data demonstrate that strains of *C. fulvum* evade *Hcr9-4E*-mediated resistance either by lack of *Avr4E* gene expression or by production of an AVR4E mutant protein that carries two amino acid substitutions, Phe^{62}Leu and Met^{73}Thr.

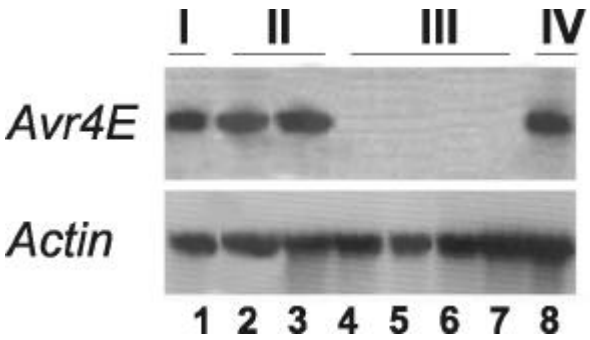

Fig. 8. Expression of *Avr4E* during growth of *C. fulvum* on susceptible tomato plants. Northern blot analysis was performed on total RNA isolated from leaves of MM-Cf0 plants that were infected with strains of *C. fulvum* that are either avirulent (I and IV) or virulent (II and III) on *Hcr9-4E*-transgenic plants. Lane 1; avirulent race 5 carrying *Avr4E*; lanes 2 and 3: virulent race 4 and strain SA carrying *avr4E*^{LT}, respectively; lanes 4-to-7: naturally virulent race 2.4, race 2.4.5.9, race 2.4.5.9.11 and race 2.4.8.11, respectively, carrying a wild-type *Avr4E* ORF; and lane 8: avirulent race 2.4.8.11 transformed with the *Avr4E* genomic sequence of race 5. The expression of *Avr4E* (upper panel) and *actin* (lower panel) was assayed using DNA fragments of either the *Avr4E* ORF or the *C. fulvum actin* gene, respectively, of which the latter served as a control probe.

The single amino acid substitution Phe^{62}Leu is sufficient for loss of AVR4E avirulence function

As Agrobacterium-mediated transient expression in tobacco has previously been demonstrated to facilitate successful expression of extracellular elicitors and membrane-anchored Cf proteins (Van der Hoorn et al., 2000), we used this method to study AVR elicitor activity. To this aim, Agrobacterium cultures carrying AVR- and Hcr9-4-encoding genes were mixed in a 1:1 ratio and infiltrated into tobacco leaves (Fig. 9A). In the presence of Hcr9-4E, but not Hcr9-4D, AVR4E induced a necrotic response, whereas AVR4 induced necrosis only in the presence of Hcr9-4D (Fig. 9A).

To investigate whether the amino acid substitutions, Phe^{62}Leu and Met^{73}Thr, are both required for loss of AVR4E avirulence function, the NIA of avr4E^{LT} and AVRE mutant proteins carrying the single amino acid substitution Phe^{62}Leu (avr4E^{L}) or Met^{73}Thr (avr4E^{T}) was examined by agroinfiltration. Therefore, a quantitative analysis (Van der Hoorn et al., 2000) was performed by infiltration of a dilution series of Agrobacterium cultures carrying AVR4E (mutant)- and Hcr9-4E-encoding genes. The NIA of AVR4 isoforms that are produced by strains of *C. fulvum* virulent on *Hcr9-4D* transgenics was also analyzed by infiltration of dilution series of Agrobacterium cultures carrying AVR4 (mutant)- and Hcr9-4D-encoding genes. The percentage of infiltrated leaf area that had become necrotic was measured 7 days post inoculation (dpi) and plotted against the percentage of Agrobacterium cultures carrying AVR (mutant)-encoding genes. For all AVR4 mutant proteins tested, a significant reduction in NIA was observed as compared to AVR4 (data not shown). These observations confirmed results obtained previously, using PVX-mediated expression (Joosten et al., 1997). Agrobacterium cultures carrying *avr4E^{LT}* induced necrosis of 50% of the infiltrated leaf area at a significantly lower dilution than Agrobacterium cultures carrying wild-type *Avr4E* (Fig. 9B). Thus, opposed to PVX-mediated expression in tomato (Fig. 7), avr4E^{LT} still exhibited some level of NIA when agroinfiltrated in tobacco leaves. Interestingly, no difference in NIA was observed between avr4E^{L} and avr4E^{LT}, or between avr4E^{T} and AVR4E (Fig. 9B), suggesting that the single amino acid substitution Phe^{62}Leu in AVR4E is sufficient to evade *Hcr9-4E*-mediated resistance.

AVR4E mutant proteins carrying single- or double amino acid substitutions are stable

We also analyzed whether the single- and double amino acid substitutions affected the protein stability of AVR4E. To test this hypothesis, we transiently expressed *Avr4E*, *avr4E^{LT}*, as well as *avr4E^{L}* and *avr4E^{T}* by agroinfiltration into tobacco leaves and analyzed the stability of the produced proteins. Moreover, to determine whether instability of AVR4

isoforms enables circumvention of *Hcr9-4D*-mediated resistance, as suggested by Joosten et al. (1997), we also tested the protein stability of these AVR4 mutant proteins.

To facilitate detection of the various mutant proteins by Western blotting, c-Myc-tagged AVR4 and AVR4E (mutant) proteins were made. Hereto, the sequence encoding AVR (mutant) proteins was cloned downstream of the sequence encoding a c-Myc-tag, which in turn is downstream of the *PR-1a* signal sequence. Co-infiltration of Agrobacterium cultures carrying c-Myc-tagged AVR-encoding genes with cultures carrying the matching *Hcr9-4* resistance gene demonstrated that the c-Myc-tag did not affect the NIA of AVR4 and AVR4E (data not shown).

Western blot analysis was performed on AF isolated from leaves that were agroinfiltrated, at opposite leaf-halves, with cultures carrying wild-type *Avr* and cultures carrying AVR (mutant)-encoding genes. Comparison of protein levels within one leaf showed that the same amount of protein was present in AF of leaves agroinfiltrated with cultures carrying AVR4E mutant-encoding genes as compared to cultures carrying wild-type *Avr4E* (Fig. 9C). However, a significantly lower amount of protein was detected for the AVR4 mutant proteins as compared to wild-type AVR4 (Fig. 9C). These data demonstrate that evasion of *Hcr9-4D*-mediated resistance is accomplished by production of AVR4 isoforms that are unstable, whereas evasion of AVR4E recognition by plants that carry *Hcr9-4E* is mediated by production of an elicitor-inactive AVR4E mutant protein that is very stable.

Discussion

The Hcr9-4E resistance protein confers resistance upon recognition of the AVR4E elicitor

Resistance trait *Cf-4* has been introgressed from wild tomato species into tomato cultivar MoneyMaker (MM) (Stevens and Rick, 1988; Boukema, 1981), resulting in the near-isogenic line MM-Cf4 that confers resistance to *Cladosporium fulvum* through recognition of the *Avr4* gene product (Joosten et al., 1994). Within the introgressed *Cf-4* region, five homologues of *Cladosporium* resistance gene *Cf-9* (*Hcr9*s) are present, of which homologue *Hcr9-4D* represents the functional *Cf-4* resistance gene. It has previously been reported that homologue *Hcr9-4E* is also a functional *Cf* gene (Takken et al., 1998) and confers resistance towards *C. fulvum* race 5 through recognition of a novel avirulence determinant, designated AVR4E (Takken et al., 1999). Here, we demonstrate that homologue *Hcr9-4E* confers the same level of resistance towards *C. fulvum* race 5 as *Hcr9-4D*. We found that the AVR4E protein is a cysteine-rich, secreted protein of 101 amino acids that is stable in

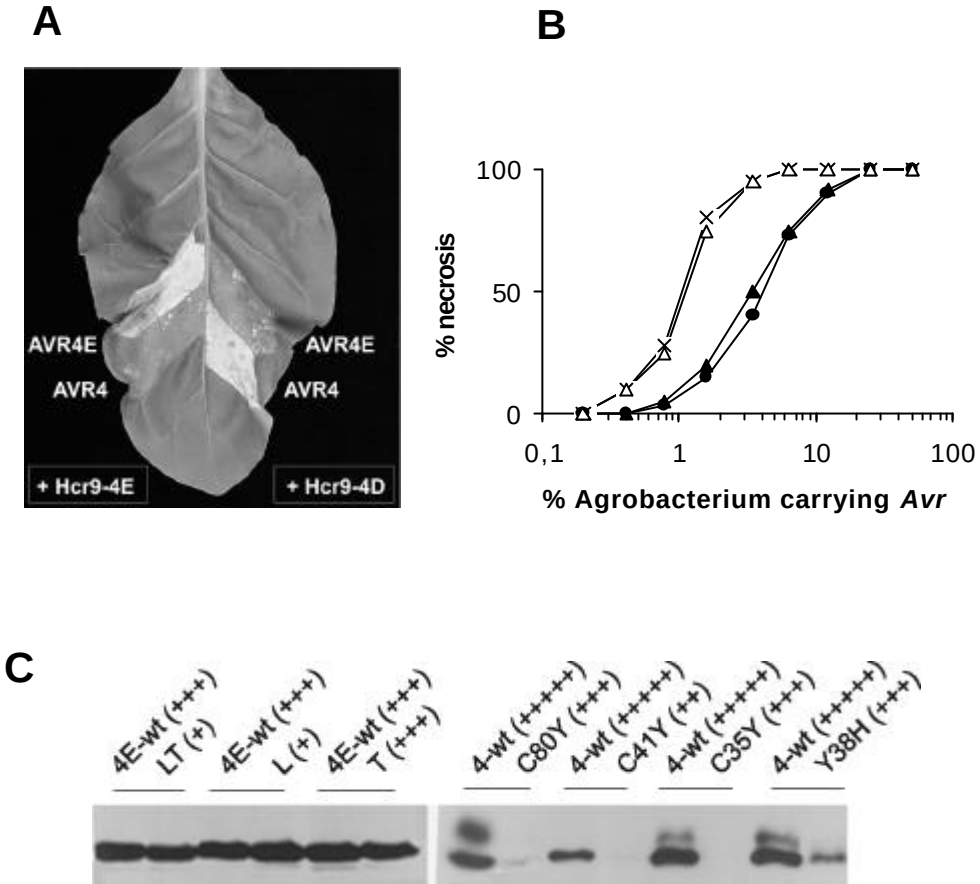

Fig. 9. Necrosis-inducing activity and stability of AVR4E and AVR4 (mutant) proteins in apoplastic fluids of agroinfiltrated tobacco leaves. **A,** Agrobacterium cultures carrying *Avr4E* or *Avr4* were co-infiltrated into leaves of 6-week-old tobacco plants in a 1:1 ratio with cultures carrying *Hcr9-4E* or *Hcr9-4D.* Necrosis-inducing activity (NIA) was scored at three days post infiltration (dpi) and photographs were taken 7 dpi. **B,** Agrobacterium cultures carrying *Avr4E* (x), *avr4E*^{T} (open triangle), *avr4E*^{L} (black triangle) or *avr4E*^{LT} (black dot) were diluted in Agrobacterium cultures carrying *Hcr9-4E* and infiltrated into tobacco leaves. The relative necrotic leaf area (percentage) was measured and plotted against the percentage of culture carrying AVR4E (mutant)-encoding genes. **C,** Western blot analysis of apoplastic fluids isolated from tobacco leaves that were infiltrated, at opposite leaf halves, with cultures carrying AVR4E/AVR4 wild-type and mutant-encoding genes. A Myc-tag fused at the N-terminus of both AVR4E- and AVR4 (mutant) proteins allowed detection of these proteins by antibodies raised against c-Myc. The NIA of the AVR4E- and AVR4 wild-type (+++++) and mutant proteins (+ and ++), as determined by agroinfiltration, is indicated between brackets.

the apoplastic space of tomato. As found for other small and stable, secreted cysteine-rich elicitor proteins of *C. fulvum* (Van den Burg et al., 2001b; Van den Hooven et al., 2001; Luderer et al., 2002b), the six cysteine residues present in AVR4E are thought to be involved in disulfide bonding. The genomic *Avr4E* sequence of race 5 was found to confer avirulence to strains that are normally virulent on *Hcr9-4E*-transgenic plants, indicating that AVR4E functions as a genuine avirulence determinant.

Circumvention of *Hcr9-4D*- and *Hcr9-4E*-mediated resistance

In order to enable growth on MM-Cf4 plants, *C. fulvum* has to evade both *Hcr9-4D*- and *Hcr9-4E*-mediated resistance. For strains of *C. fulvum* virulent on *Hcr9-4D*-transgenic plants, a variety of single point mutations in the open reading frame (ORF) of the *Avr4* gene has been found (Joosten et al., 1997). It appeared that these modifications resulted in unstable AVR4 isoforms, as previously has been suggested by the authors, indicating that protein stability plays a crucial role in evasion of *Hcr9-4D*-mediated resistance. Unlike AVR4, circumvention of AVR4E recognition by plants that carry *Hcr9-4E* is mediated by production of a stable AVR4E mutant protein. This AVR4E mutant protein carries two amino acid substitutions, Phe^{62}-to-Leu and Met^{73}-to-Thr ($avr4E^{LT}$), as a result of a double point mutation in the *Avr4E* ORF. No other modifications were identified in the *Avr4E* ORF of strains that are virulent on *Hcr9-4E* transgenics. PVX-mediated expression of $avr4E^{LT}$ in tomato plants that express *Hcr9-4E* induced no systemic necrosis, whereas Agrobacterium-mediated expression of $avr4E^{LT}$ in tobacco leaves showed some level of necrosis-inducing activity (NIA). The higher level of sensitivity towards elicitor proteins observed in tobacco is thought to be due to a more efficient *Cf*-mediated defense-signaling pathway in tobacco as compared to tomato (Hammond-Kosack et al., 1998; Kamoun et al., 1999a). Nevertheless, it appeared that production of $avr4E^{LT}$ mutant protein does not trigger *Hcr9-4E*-mediated defense responses that restrict growth of *C. fulvum* .

We further analyzed whether amino acid substitutions Phe^{62}Leu and Met^{73}Thr are both required for loss of AVR4E avirulence function. Interestingly, the AVR4E mutant protein carrying Met^{73}Thr ($avr4E^{T}$) appeared to be as active as AVR4E, whereas mutant protein carrying Phe^{62}Leu ($avr4E^{L}$) is as active as $avr4E^{LT}$, suggesting that the single amino acid substitution Phe^{62}Leu is sufficient to evade *Hcr9-4E*-mediated resistance. Moreover, as demonstrated for $avr4E^{LT}$, the $avr4E^{L}$ and $avr4E^{T}$ mutant proteins are as stable as the wild-type AVR4E protein. Thus, *Hcr9-4E*-mediated resistance is evaded by secretion of an AVR4E mutant protein that exhibits an essential conformational- or qualitative change, as a result of amino acid substitution Phe^{62}Leu.

For eight other strains of *C. fulvum* virulent on *Hcr9-4E*-transgenic plants that we analyzed, no modifications were found in the *Avr4E* ORF. Nevertheless, these strains do not secrete a functional AVR4E elicitor protein. Strikingly, it appeared that no AVR4E protein was produced during growth of these strains in tomato leaves due to the lack of *Avr4E* gene expression. Transformation with the genomic *Avr4E* sequence of *C. fulvum* race 5 resulted in accumulation of *Avr4E* transcripts *in planta*, thereby conferring avirulence to the fungus on plants that carry *Hcr9-4E.* Thus, it seems that all elements required for proper *Avr4E* expression, including transcription factors and regulatory proteins, are present in parent strains that lack *Avr4E* expression. Possibly, the lack of *Avr4E* gene expression in these virulent strains is caused by a defect in (cis-regulatory elements of) the promoter of *Avr4E.* Also, for the rice blast fungus, *Magnaporthe grisea*, it has been reported that *PWL4* is a non-functional *Avr* gene due to a defective promoter, as expression of the *PWL4* ORF under control of the *PWL1* or the *PWL2* promoter conferred avirulence to the fungus on weeping lovegrass (Kang et al., 1995). In addition, insertion of a Pot3 transposon into the promoter of the *AVR-Pita* gene of *M. grisea* resulted in gain of virulence on rice cultivars carrying the *Pi-ta* resistance gene (Kang et al., 2001). Moreover, in the promoter sequence of the *PDA1* gene of *Nectria heamatococca* positively acting elements are present that are necessary for high level of transcription *in vitro* (RuanandStraney, 1996). In the promoter of *Avr4E,* (TA)GATA boxes and CAAT-like boxes are present that might regulate nitrogen metabolism or conidiophore development, respectively, upon binding of transcription factors like AreA (AdrianopoulosandTimberlake, 1994; Snoeijers et al., 1999; Pérez-García et al., 2001). Strains that lack *Avr4E* expression might carry modifications in the promoter, possibly in elements that are essential for *Avr4E* expression. On the other hand, lack of *Avr4E* expression might be caused by a transposon insertion in the promoter of *Avr4E*, as found in the *Avr2* ORF of strains of *C. fulvum* virulent on *Cf-2* plants (Luderer et al., 2002b). Alternatively, the terminator region of *Avr4E* might carry mutations, resulting in *Avr4E* transcripts that are unstable.

Sequence polymorphism in the ORF of *Avr* genes

As avr4E^{L} exhibits the same elicitor activity as avr4E^{LT}, one would expect that the single amino acid substitution Phe^{62}Leu would be sufficient for loss of AVR4E avirulence function. However, thus far, no strains of *C. fulvum* virulent on *Hcr9-4E*-transgenic plants have been identified that carry *avr4E^{L}*. Moreover, although avr4E^{T} is as active as AVR4E, no strains avirulent on *Hcr9-4E* transgenics were found that carry this mutation. This implies that strains carrying *avr4E^{LT}* originated from strains carrying wild-type *Avr4E* as a result of one single evolutionary event. However, another, more favorable, hypothesis would be that two

independent evolutionary events occurred, where initially strains evolved that carried *avr4E*^{T}, which are still avirulent on plants carrying *Hcr9-4E,* followed by emergence of strains that carry *avr4E*^{LT}, which are virulent on these plants.

Within the *C. fulvum* population, several strains of *C. fulvum* have been identified that evade more than one *Cf*-mediated resistance trait, including *Cf-2, Cf-4 ("Hcr9-4D"), Cf-5, Cf-9,* and *Hcr9-4E* (see also Chapter 6). Strains virulent on plants that express *Cf-9* lack the entire *Avr9* ORF (Van Kan et al., 1991). The ORF of the *Avr2* and *Avr4* genes, however, contain more sequence variation than the *Avr4E* gene (Joosten et al., 1997; Luderer et al., 2002b), suggesting that *C. fulvum* has endured more extensive selection pressure to evade *Cf-2*- and *Hcr9-4D*-mediated resistance than to evade *Hcr9-4E*-mediated resistance. However, a total of 17 breeding lines that carry *Cf-4* were also found to carry *Hcr9-4E* (Haanstra et al., 2000), implying that selection pressure to evade AVR4 recognition is identical to that of AVR4E. Moreover, a low genetic variability was found within the *C. fulvum* population, as determined by AFLP analysis, implying that the *C. fulvum* population consists of a single clonal lineage (Joosten and De Wit, 1999). Thus, strains of *C. fulvum* virulent on *Hcr9-4D*-transgenic plants might have evolved from a common ancestor strain that already enabled the ability to grow on plants carrying *Hcr9-4E.*

The intrinsic function of AVR4E for *C. fulvum*

Strains of *C. fulvum* that carry *avr4E*^{LT} or strains that lack *Avr4E* expression do not show a measurable reduction in virulence on susceptible tomato plants when compared to strains that carry wild-type *Avr4E*, suggesting that AVR4E does not clearly contribute to virulence of *C. fulvum*. On the other hand, both AVR4E and avr4E^{LT} might play a role in virulence of *C. fulvum.* As only one type of modification was found in the *Avr4E* ORF, mutations other than Phe^{62}Leu and Met^{73}Thr might affect the stability of AVR4E and thereby the virulence of *C. fulvum.* Moreover, although for various virulent strains no *Avr4E* transcripts could be detected on Northern blots, there could still be some level of *Avr4E* expression present that is below detection level. This might result in the presence of minute amounts of potentially stable AVR4E protein, which would be sufficient for full virulence of *C. fulvum* on susceptible tomato plants. Thus, as previously demonstrated for *Phythophtora parasitica* isolates (Colas et al., 2001), downregulation rather than a complete loss of *Avr4E* expression might constitute a suitable mechanism for *C. fulvum* to evade *Hcr9-4E*-mediated resistance, without deleterious consequences for the pathogen.

Materials and Methods

Plants and strains of *Cladosporium fulvum*

Tomato (*Lycopersicon esculentum*) cultivar Moneymaker (MM), near isogenic line MM-Cf4 (containing an introgression segment that carries the *Cf-4* locus), transgenic tomato plants that carry *Hcr9-4D* or *Hcr9-4E* (Thomas et al., 1997), *Nicotiana clevelandii* and (transgenic) tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) plants, were grown under standard greenhouse conditions. The various strains of *Cladosporium fulvum* were cultured according to De Wit and Flach (1979).

C. fulvum inoculation procedure and isolation of apoplastic washing fluids

Strains of *C. fulvum* were inoculated onto tomato plants as described by De Wit (1977). Briefly, suspensions of conidia of *C. fulvum* (approximately 5 x 10^6 conidia per ml) were used to inoculate the lower leaf surface of 3-to-6-week-old tomato plants. Apoplastic-washing fluids (AFs) were isolated from *C. fulvum*-infected tomato leaves, three weeks after inoculation, as described by De Wit and Spikman (1982).

DNA manipulations

All plasmid manipulations were carried out essentially as described by Sambrook et al. (1989). Polymerase chain reactions (PCRs) were performed with *Pfu* (Stratagene, La Jolla, CA) or with the Expand High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Restriction enzymes, T4 ligase, and *Escherichia coli* DH5á cells were obtained from Invitrogen (Breda, The Netherlands). Oligonucleotides were synthesized by Amersham-Pharmacia (Buckinghamshire, UK) and probes were labeled with the Random Primers DNA labeling System (Promega, Breda, The Netherlands), using α^{32}P-dATP (Amersham-Pharmacia, Buckinghamshire, UK).

Purification of the AVR4E elicitor protein

Proteins present in AF isolated from a compatible interaction between *C. fulvum* race 5 and MM-Cf0 were separated by gel filtration on a Sephadex G-50 column (Amersham-Pharmacia, Roosendaal, The Netherlands), as described by Joosten et al. (1990). The fractions that showed specific necrosis-inducing activity (NIA) when injected into leaves of *Hcr9-4E*-transgenic plants were subjected to anion exchange chromatography on a Resource Q column (Amersham-Pharmacia, Roosendaal, The Netherlands), according to the protocol described by Laugé et al. (2000). Fractions that are biologically active were subsequently loaded on a ProRPC HR 5/10 reverse-phase column (Amersham-Pharmacia,

Roosendaal, The Netherlands), as described by Joosten et al. (1994). A highly purified protein that exhibits NIA in leaves of *Hcr9-4E* plants was obtained and used to determine an N-terminal and an internal amino acid sequence of the AVR4E protein.

Isolation of a cDNA and a genomic clone of *Avr4E*

To facilitate isolation of the *Avr4E* gene by reverse genetics, degenerated oligonucleotides were designed. Two forward primers N1 (5'-gtnggnaaycargcngartgg-3') and N2 (5'-gtngayggnacngcnathcc-3') were constructed, based on the N-terminal amino acid sequence (VGNQAEW and VDGTAI), respectively. Reverse primer X-3b (5'-gcnccnacytcdatytccca-3') was designed, based on the internal protein sequence (WEIEVGG). Anchored PCR was performed essentially according to Stuurman et al. (1996), using *Bst*YI and *Apo*I to digest 100 ng of genomic DNA of *C. fulvum* race 5, followed by ligation of the corresponding double-stranded oligonucleotides ("anchors"). A series of nested PCR reactions were performed with N1 and N2 together with primers VECT24 (5'-agcactctccagcctctcaccgcc-3', which anneals to the *Bst*YI-digested anchor site) and RH24 (5'-agcactctccagcctctctcaccgca-3', which anneals to the *Apo*I-digested anchor site), and the PCR products were sequenced. Sequence analysis revealed that the putative translation products of the PCR fragments correspond to the AVR4E peptide sequences. Additional 5'-sequence information was obtained by PCR using primer RH24 together with reverse primers X-3b, as well as by PCR using RH24 together with two homologous reverse primers N3 (5'-cccagcacgttcaagaac-3') and N4 (5'-gaagcagtcacagaggctg), which anneal to the 5'-end of *Avr4E.*

In order to isolate the full-length *Avr4E* sequence, a cDNA library derived from a compatible interaction between *C. fulvum* race 5 and tomato (Van Kan et al., 1991) was screened using the *Avr4E* PCR fragments as a probe, according to the method described by Van den Ackerveken et al. (1992). This screening resulted in the isolation of a clone carrying the full-length *Avr4E* open reading frame (ORF). By screening a genomic library derived from *C. fulvum* race 4, we obtained promoter- and terminator sequence information, which allowed subsequent isolation of the genomic *Avr4E* sequence of *C. fulvum* race 5 by PCR, using the following primers; forward primer PROM-B (5'-cgcggatcctaactctagggtctacc-3', *BamH*I-site underlined) and reverse primer TER-X (5'-gatcctcgaggccacctatgcataacttg-3', *Xho*I-site underlined). PCR resulted in a fragment that contains a promoter region of 1475 bp and a terminator region of 190 bp, flanking the *Avr4E* ORF of *C. fulvum* race 5. The PCR product was digested with *BamH*I and *Xho*I and ligated into *Bgl*II/*Xho*I-digested site of pAN7-1, a plasmid carrying a hygromycin B selection marker (Punt et al., 1987). The obtained plasmid was used to transform strains of *C. fulvum* that are virulent on *Hcr9-4E*-transgenic plants.

Transformation of *C. fulvum*

The procedure for preparation of *C. fulvum* protoplasts was adapted from the method described by Harling et al. (1988) and Van den Ackerveken et al. (1992). Mycelium of *C. fulvum* strain SA, race 4, race 2.4.8.11, and race 2.4.5.9 was harvested from 3-day-old cultures that were grown *in vitro* in liquid B5 medium. Protoplast were obtained upon digestion of the mycelium in MM (20 mM 2-[*N*-morpholino] ethanesulfonic acid, pH 5.8, 1 M $MgS0_4$), containing 25 mg of Glucanex (Novo Nordisk, Denmark) and 1 mg of kitalase (Wako Pure Chemical Inductries, Ltd.) per ml. Transformation of the protoplasts was achieved with polyethylene glycol 6000 (Merck), according to Oliver et al. (1987). Transformants that were resistant to hygromycin B (Sigma, Bornem, Belgium), obtained 3-4 weeks after culture on potato dextrose agar medium were subcultured to obtain mono-spore isolates.

Sequencing the open reading frame of *Avr4E* in virulent and avirulent strains of *C. fulvum*

For standard PCR analysis, genomic DNA of *C. fulvum* was isolated according to the procedure described by Cenis (1992), after growth of the fungus for 5 days in liquid B5 medium. Following DNA isolation, the *Avr4E* ORF was PCR-amplified using forward primer 4E-F (5'- ggacgagtcttcgaagga-3'), which anneals 76 nucleotides upstream from the ATG start codon, and reverse primer 4E-R3 (5'- ctgagattagaaggtagttag-3'), annealing 51 bases downstream of the TAG stop codon. Sequence analysis of the *Avr4E* ORF was also performed using the 4E-F and 4E-R3 primers.

Northern blot analysis

Total RNA from *C. fulvum*-infected tomato leaves was isolated 21 days after inoculation using a hot phenol-extraction procedure, according to the manufacturer's instructions (CLONTECH Laboratories, Palo Alto, USA). Fifteen μg of glyoxal-denatured RNA was separated on 0.01 M sodium phosphate pH 7.0 agarose gels and transferred to Hybond N^+ membrane (Amersham-Pharmacia, Buckinghamshire, UK) by capillary blotting with 0.025 M sodium phosphate buffer pH 7.0 (Sambrook et al., 1989). The RNA was immobilized by UV-crosslinking and the RNA gel blots were prehybridized at 65°C for 60 min in modified Church and Gilbert buffer (0.5 M phosphate buffer, pH 7.2, 7% SDS and 1 mM EDTA) (Church and Gilbert, 1984). Subsequently, the blots were hybridized over-night at 65°C in the same buffer containing radio-labeled DNA fragments, representing the *Avr4E* gene and the actin gene of *C. fulvum*. The blots were washed at 65°C with 0.5 x SSC (75 mM NaCl, 7.5 mM sodium

citrate), containing 0.1 % SDS. Kodak X-OMAT films were subsequently exposed to the blots.

Construction of PVX derivatives and PVX inoculation procedure

The recombinant constructs PVX::*Avr4E* and PVX::*avr4E*^{LT} were obtained by PCR using genomic DNA isolated from *C. fulvum* race 5 and race 4, respectively, as templates. Constructs that carry the endogenous signal sequence of *Avr4E* for extracellular targeting were obtained by PCR with forward primer PVX4E-N (5'-ccatcgatgcagttttccaacccctca-3') and reverse primer PVX4E-R (5'-ccatcgatctatctgtttgccatcctctc-3') (*Cla*I site underlined). PVX constructs carrying the *PR-1a* signal sequence (Hammond-Kosack et al., 1995), upstream of the sequence encoding mature AVR4E (mutant) protein, were obtained by PCR-mediated overlap extension. First, to obtain the *PR-1a* signal sequence with an *Avr4E* overhang at the 3'-end, PCR was performed using forward primer OX10 (5'-caatcacagtgttggcttgc-3') and reverse primer PR4E-R (5'-gcgcgagaaatcggcacggcaagagtggg-3'), together with PVX::*Avr4* (Joosten et al., 1997) as a template. The sequence encoding mature AVR4E and avr4E^{LT} were obtained by PCR using forward primer 4E-F (5'-tcttgccgtgccgatttctcgcgcgattgc-3') and PVX4E-R, together with genomic DNA of race 5 and race 4, respectively, as a template. The two sets of PCR products were subsequently used as templates in an overlap extension PCR with primers OX10 and PVX4E-R. The PCR fragments were digested with *Cla*I and cloned into the *Cla*I-digested site of PVX-expression vector pTXΔGC3A (Hammond-Kosack et al., 1995), downstream of the PVX coat protein promoter. *In vitro* transcription, amplification of the virus particles on *Nicotiana clevelandii* and PVX inoculations on (transgenic) tomato plants were performed as described by Hammond-Kosack et al. (1995).

Construction of binary plasmids and transformation of *Agrobacterium*

Binary plasmids carrying *Avr4E* and *Avr4* and their derivatives were constructed as follows: PCR fragments containing the ORF of *Avr4E* and *avr4E*^{LT} were generated using AT4E-N (5'-tagctcgagcgatttctcgcgcgattgcc-3', *Xho*I site underlined) and AT4E-B (5'-cgcggatcctatctgtttgccatcctctc-3', *BamH*I site underlined) with PVX::*Avr4E* and PVX::*avr4E*^{LT} vectors as a template, respectively. Fragments containing the sequences that encode mature avr4E^{L} and avr4E^{T} were obtained by overlap extension PCR with PVX::*Avr4E* as a template. For *avr4E*^{L}, the following sets of primers were used: AT4E-N with F^{62}L-R (5'-cacaagaacagctctctc-3') and F^{62}L-F (5'-gagagagctgttcttgtg-3') with AT4E-B. For *avr4E*^{T}, AT4E-N with M^{73}T-R (5'-cgactccgacgtccgcgc-3') and M^{73}T-F (5'-gcgcggacgtcggagtcg-3') with AT4E-B were employed. PCR fragments containing the ORF of *Avr4*, *avr4 (C*^{35}*Y), avr4 (Y*^{38}*H), avr4 (C*^{41}*Y)* and *avr4 (C*^{80}*Y)* (Joosten et al., 1997) were generated using primer A4-

NX (5'-tagctcgagcaaggccccaaaactcaacc-3', *Xho*I site underlined) and A4-BC (5'-cgcggatccctattgcggcgtctttaccg-3', *BamH*I site underlined) with genomic DNA of *C. fulvum* race 5, race 2.4.8.11 ($C^{35}Y$), race 4 ($Y^{38}H$), race 4 ($C^{41}Y$) and race 2.4.5.9.11 ($C^{80}Y$) as templates. The amplified fragments were digested with *Xho*I/*BamH*I, cloned into the *Xho*I/*BamH*I-digested site of pBluescript SK^+ (Amersham-Pharmacia, Buckinghamshire, UK) and sequenced. This plasmid was digested with *Xho*I and *BamH*I, and the fragments that contain the *Avr* ORFs were subsequently cloned into the *Xho*I/*BamH*I-digested site of binary plasmid pNW30, a derivative of pRH271 (Van der Hoorn, unpublished). This binary plasmid pNW30 carries the Cauliflower Mosaic Virus (CaMV) 35S-promoter and the PI-II terminator (An et al., 1989), both of which flank the *PR-1a* signal sequence (Hammond-Kosack et al., 1995) and the sequence encoding a double c-Myc-tag.

Binary plasmid carrying *Hcr9-4E* was constructed by PCR with primers Cf 4E-F (5'-agctccatgggttgtgtaaaacttatatttttcatgc-3') and Cf 4E-R (5'-agctctgcagctaatatatcttttcttgtgctttttt-cattctcg-3'), using a genomic clone containing *Hcr9-4E* (Takken et al., 1998) as a template, following the procedure as described for binary plasmid carrying *Hcr9-4D* (pCf-4) constructed by Van der Hoorn et al. (2000).

The binary plasmids were transferred to *Agrobacterium tumefaciens* strain MOG101 (Hood et al., 1993) by electroporation.

Agroinfiltration and analysis of apoplastic washing fluids

Agrobacterium-mediated transient expression was performed essentially as described by Van der Hoorn et al. (2000). Cultures containing recombinant Agrobacterium carrying the different binary plasmids were resuspended to a final OD_{600} of 2 and infiltrated into tobacco leaves in the presence of 200 μM acetosyringone. As tobacco leaves at different developmental stages might exhibit different transient expression levels, the stability of the AVR (mutant) proteins was determined within one tobacco leaf. Three-to-four days after co-infiltration with Agrobacterium carrying *Hcr9-4D* or *Hcr9-4E*, the necrosis-inducing activity of the AVR4 and AVR4E (mutant) proteins was scored. AF was isolated three days after infiltration with cultures that carry AVR-encoding genes, according to the method described by De Wit and Spikman (1982). Ten times concentrated AF (7 μl) was supplied with 2 μl 5x SDS loading buffer (50 mM Tris-HCl pH 6.8, 5% (w/v) SDS, 10% (v/v) glycerol, 5% 2-mercaptoethanol and 0.0025% bromophenol blue) and incubated at 95°C for 5 min. Proteins were separated by SDS-PAGE on gels containing 15% (w/v) acrylamide (Laemmli, 1970) and transferred to nitrocellulose membranes (Schleider and Schuell, Dassel, Germany) by blotting for 2 hrs at 250 mA. Detection of c-Myc-tagged proteins was performed by incubation of the filters in blocking buffer (BB) (1x phosphate-buffered saline (PBS) (Oxoid)

and 5% skimmed milk powder [ELK, Campina]), followed by over-night incubation in BB supplied with 1:1000 diluted antibodies raised against c-Myc (rabbit polyclonal IgG [Santa Cruz Biotechnology]). After 3 x 15 min washes in wash buffer (WB) (1x PBS, 0.05% Tween-20), filters were incubated for 2 hrs in BB supplied with a 1:1000 diluted secondary antibody (Anti-Rabbit Ig-horseradish peroxidase [Amersham-Pharmacia, Buckinghamshire, UK]). Following 3 x 15 min washes in WB, c-Myc-tagged proteins were detected using Super Signal Chemiluminescent Substrate (Pierce, Rockford, USA) and Kodak X-OMAT films. Immunodetection of ECP2 proteins was performed according to the method described by Wubben et al. (1994).

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Chapter 3

The AVR4 elicitor protein of *Cladosporium fulvum* binds to fungal components with high affinity

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* Equal author contribution

The AVR4 elicitor protein of *Cladosporium fulvum* binds to fungal components with high affinity

Abstract

The interaction between tomato and the fungal pathogen *Cladosporium fulvum* complies with the gene-for-gene system. Strains of *C. fulvum* that produce race-specific elicitor AVR4 induce a hypersensitive response, leading to resistance, in tomato plants that carry the *Cf-4* resistance gene. The mechanism of AVR4 perception was examined by performing binding studies with ^{125}I-AVR4 on microsomal membranes of tomato plants. We identified an AVR4-high affinity-binding site (K_D = 0.05 nM), which exhibited all the characteristics expected for ligand-receptor interactions, such as saturability, reversibility and specificity. Surprisingly, the AVR4-high affinity-binding site appeared to originate from fungi present on infected tomato plants rather than from the tomato plants themselves. Detailed analysis showed that this fungus-derived, AVR4-specific binding site is heat and proteinase K resistant. Affinity crosslinking demonstrated that AVR4 specifically binds to a component of about 75 kDa that is of fungal origin. Our data suggest that binding of AVR4 to fungal component(s) is related to the intrinsic virulence function of AVR4 for *C. fulvum*.

Introduction

Active disease resistance in plants relies on a highly sensitive and specific defense system that enables recognition and evasion of invading pathogens. Pathogens that produce nonspecific elicitors are able to initiate a disease response in most genotypes within a plant species (Knogge 1996). Race-specific elicitors are considered to induce resistance only in genotypes of host plants that carry the corresponding resistance (*R*) gene (Flor 1971; De Wit 1997). Addition of elicitor proteins to suspension-cultured plant cells results in a rapid induction of plant defense-associated responses (Ham et al. 1991; Basse et al. 1993; Nürnberger et al. 1994; Boller 1995; Hanania and Avni 1997; Meindl et al. 1998; Felix et al. 1999; Lee et al. 2001). The high sensitivity of suspension-cultured cells and their extremely quick responses to low amounts of elicitor, suggest that the induction of defense responses is mediated by receptors that exhibit high affinities for elicitor proteins. Indeed, specific high affinity-binding sites (HABSs) have been identified for elicitor proteins, peptides and oligosaccharide signals (Cheong and Hahn 1991; Nürnberger et al. 1995; Mithöfer et al.

1996; Ito et al. 1997; Matsubayashi et al. 1997; Bourque et al. 1999; Matsubayashi and Sakagami 1999; Scheer and Ryan 1999; Meindl et al. 2000). Since the majority of HABSs identified thus far resides in the plasma membrane of plant cells, a role for HABS as putative receptors for elicitor molecules has been proposed. A close correlation has also been found between the biological activity of elicitor molecules and the affinity for their binding sites, which further implies that the HABSs for these elicitors act as functional receptors (Hanania and Avni 1997; Meindl et al. 1998 and 2000; Thevissen et al. 2000).

The interaction between tomato and the extracellular, biotrophic pathogen *Cladosporium fulvum* is a well-established model system that complies with the gene-for-gene model. Strains of *C. fulvum* that carry avirulence (*Avr*) genes induce an active defense response, including the hypersensitive response (HR), in tomato plants that carry the corresponding *Cf* resistance genes (Joosten and De Wit 1999). Several *Cf* genes and their corresponding *Avr* genes have been cloned (Joosten and De Wit 1999). The *Cf* gene family encodes putative plasma membrane-anchored proteins with a predicted extracellular leucine-rich repeat domain, a transmembrane region and a short cytoplasmic tail (Thomas et al. 1998). For *Cf-4* and *Cf-9*, the corresponding avirulence genes of *C. fulvum*, *Avr4* and *Avr9,* respectively, have been cloned (Van den Ackerveken et al. 1992; Joosten et al. 1994). AVR4 and AVR9 elicitor proteins are both encoded as pre-proproteins that are processed by fungal and plant proteinases within the leaf apoplast into mature proteins of 86 and 28 amino acids, respectively. To date, very little is known about the intrinsic function of AVR4 and AVR9 for the fungus. No clear deleterious effects on virulence have been found in strains of *C. fulvum* in which either the *Avr4* or the *Avr9* gene is truncated or deleted (Marmeisse et al. 1993; Joosten et al. 1997). Nevertheless, both *Avr4* and *Avr9* gene products may have a role in virulence, development, reproduction, or general fitness of *C. fulvum.*

The predicted extra-cytoplasmic localization of Cf-4 and Cf-9 would fit with a model in which the secreted AVR4 and AVR9 proteins are perceived in the leaf apoplast. Suspension-cultured tobacco cells expressing *Cf-9* rapidly produce defense response-associated active oxygen species in response to AVR9 (Piedras et al. 1998). However, no direct interaction between AVR9 and Cf-9 has yet been demonstrated (Luderer et al. 2001b). Furthermore, binding studies with AVR9 have shown that both MM-Cf9 (resistant) and MM-Cf0 (susceptible) plants contain an AVR9-specific HABS in the plasma membrane (Kooman-Gersmann et al. 1996), suggesting an indirect role for Cf-9 in perception of AVR9. Such indirect perception has been referred to as the ‘Guard hypothesis’ (Van der Biezen and Jones 1998), whereby it is proposed that the HABS represents a virulence target for AVR9. The Cf-9 protein is thought to act as a ‘guard’ to monitor the behavior of a host-

encoded virulence protein or proteins that are targets for AVR9 (Luderer and Joosten 2001a; Van der Hoorn et al. 2002).

In this study, we have investigated the binding of AVR4 to microsomal membranes of MM-Cf4 (resistant) and MM-Cf0 (susceptible) tomato plants, and non-host plant species. We have identified an AVR4 HABS that appeared to originate from fungi present on infected tomato plants rather than from tomato itself. Interestingly, this fungus-derived AVR4 HABS is heat and proteinase K resistant. In addition, affinity crosslinking demonstrated that AVR4 specifically interacts with a component of approximately 75 kDa that is of fungal origin. Binding of AVR4 to fungal components in relation to its intrinsic virulence function for *C. fulvum* will be discussed.

Results

Production and biological activity of iodinated AVR4

In order to examine whether a high affinity-binding site (HABS) is present for AVR4 on tomato membranes, it was essential to obtain large quantities of pure, elicitor-active AVR4 protein. The methylotrophic yeast *Pichia pastoris* was used as an eukaryotic, heterologous expression system (Romanos 1995) to obtain high levels of correctly folded mature AVR4 protein. AVR4 protein isolated from intercellular fluids (IF) obtained from *C. fulvum*-infected tomato leaves is unglycosylated (Joosten et al. 1997). To prevent yeast-specific glycosylation of AVR4; the potential *N*-linked glycosylation site (NLS) in AVR4 was removed by changing the codon for serine into a codon for alanine in the open reading frame (ORF) of *Avr4*. By using *Potato virus X* (PVX)-mediated expression in MM-Cf4 plants, as described by Joosten and associates (1997), this mutation was found not to affect the necrosis-inducing activity of AVR4 (data not shown). Heterologously expressed AVR4 protein was purified from the culture filtrate by ion exchange and reverse phase high-performance liquid chromatography (HPLC) (Van den Burg et al. 2001). Injection of purified AVR4 into MM-Cf4 leaves resulted in a clear hypersensitive response (HR), demonstrating that the protein had retained its elicitor activity (Van den Burg et al. 2001).

To test whether iodination affects the biological activity of AVR4, iodination with non-radioactive iodine (^{127}I) was performed using a lactoperoxidase labeling protocol, resulting in mono ^{127}I-labeled AVR4. Unlabeled AVR4 induces a rapid oxidative burst in suspension-cultured tobacco cells expressing *Cf-4* in a concentration-dependent manner (De Jong et al. 2002). Addition of ^{127}I-labeled AVR4 protein to these cell suspensions resulted in a similar, concentration-dependent response (Fig. 1). This demonstrates that iodination does not affect the biological activity of the AVR4 elicitor. Identical labeling conditions were applied

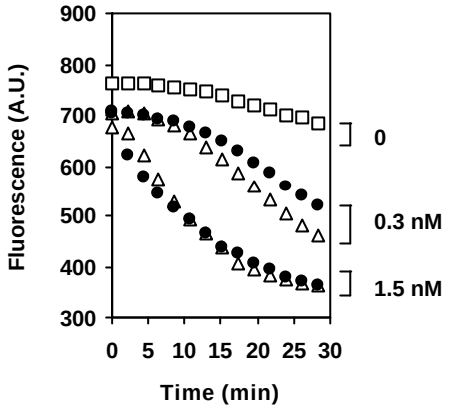

Fig. 1. Oxidative burst of suspension-cultured tobacco cells expressing *Cf-4* after treatment with AVR4 or ^{127}I-AVR4. Suspension-cultured tobacco cells expressing *Cf-4* were supplied with the fluorescent probe pyranin (10µg/ml) and challenged with buffer (open square) and with two concentrations (0.3 and 1.5 nM) of unlabeled AVR4 (black dot) or ^{127}I-labeled AVR4 (open triangle). Quenching of the pyranin fluorescence, due to production of H_2O_2 by the cells, was measured in time. A.U. = arbitrary units.

with radioactive iodine (^{125}I) to obtain mono ^{125}I-labeled AVR4 that was used in subsequent binding experiments.

Binding of ^{125}I-AVR4 to microsomal membranes of MM-Cf4 tomato plants

To examine the presence of AVR4-specific binding sites on microsomal membrane fractions (MFs) isolated from MM-Cf4 tomato leaves, binding of ^{125}I-AVR4 to MFs was followed over time and at various temperatures. Optimal binding was achieved at 37°C (data not shown). An AVR4-specific binding site was found and binding reached equilibrium 4 h after incubation with AVR4 at 37°C (Fig. 2A). Specific binding of ^{125}I-AVR4 remained stable up to 8 h, followed by a slow, partial decrease after 22 h of incubation (data not shown). At lower temperatures, i.e. 4°C and 24°C, equilibrium of ^{125}I-AVR4-specific binding was reached after 22 h and 10 h of incubation, respectively (data not shown).

Addition of a 500-fold molar excess of unlabeled AVR4 at equilibrium resulted in a decrease of specific binding of ^{125}I-AVR4, demonstrating that AVR4-specific binding is reversible (Fig. 2B). From these data, a dissociation constant (K_D) of 0.09 nM was calculated for AVR4 (Fig. 2C), suggesting that AVR4 binds to microsomal membranes of MM-Cf4 with high affinity.

Saturation of ^{125}I-AVR4 binding to microsomal membranes of MM-Cf4

Incubation of MFs isolated from MM-Cf4 leaves with increasing concentrations of ^{125}I-AVR4 showed that AVR4 binding is saturable (Fig. 3A). Saturation of AVR4-specific binding was reached at an ^{125}I-AVR4 concentration of approximately 0.25 nM (Fig. 3A). Scatchard analysis performed on these data resulted in a plot with a linear slope, which implies a uniform population of AVR4-specific binding sites. The calculated K_D value of 0.05 nM (K_D =

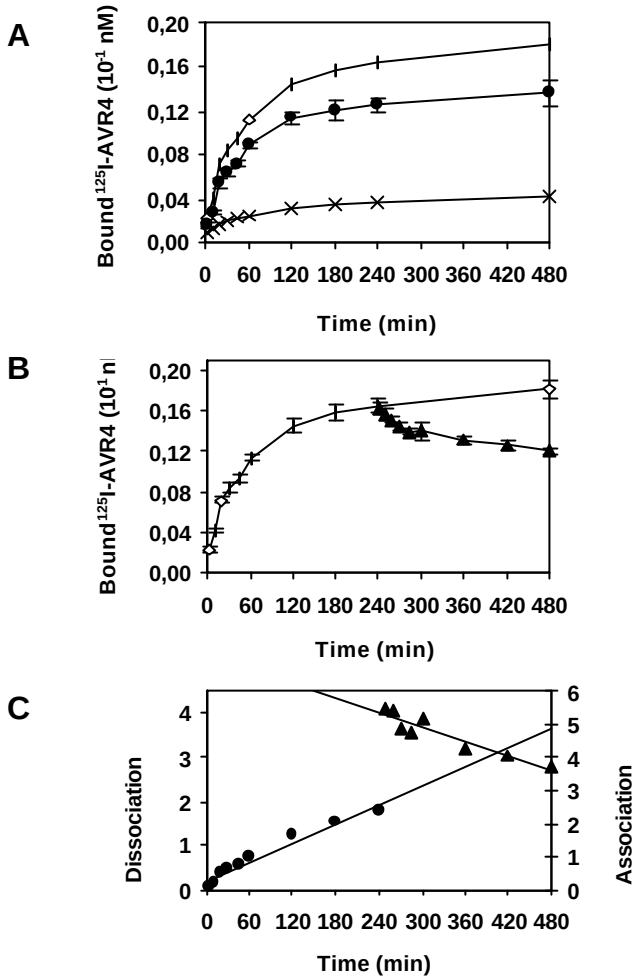

Fig. 2. Binding of ^{125}I-AVR4 to microsomal membranes of MM-Cf4 tomato. Microsomal membrane fractions of MM-Cf4 leaves were incubated with 0.07 nM ^{125}I-AVR4 at 37°C over different time points. Each data point represents the average of six independent binding assays. A, Time course of AVR4 binding was assayed in the absence (open diamond) or in the presence (x) of a 500-fold molar excess of unlabeled AVR4, which was added at t = 0 min. Specific binding (black dot) was determined by subtracting aspecific binding (x) from total binding (open diamond). B, Reversibility of AVR4 binding was assayed by adding a 500-fold molar excess of unlabeled AVR4 at t = 240 min (black triangle). C, Kinetics of specific association (-ln [1 - B/B_{max}]) (black dot) and dissociation (ln [cpm of B]) (black triangle) calculated from A and B (Hulme, 1999). The dissociation rate constant of the receptor-ligand complex (k_{off} = -slope) is 5.4 x 10^{-3} min^{-1} and the dissociation half-time ($t_{1/2}$ = ln2/ k_{off}) is 128 min. The association rate constant (k_{on} = [slope - k_{off}]/[^{125}I-AVR4]) is 0.063 nM^{-1} min^{-1}, and the half-time ($t_{1/2}$ = ln2/ [k_{on} x (^{125}I-AVR4) + k_{off}]) is 72 min. The ratio k_{off} / k_{on} yields a dissociation constant (K_D) of 0.09 nM. B = specific binding and B_{max} = specific binding at equilibrium.

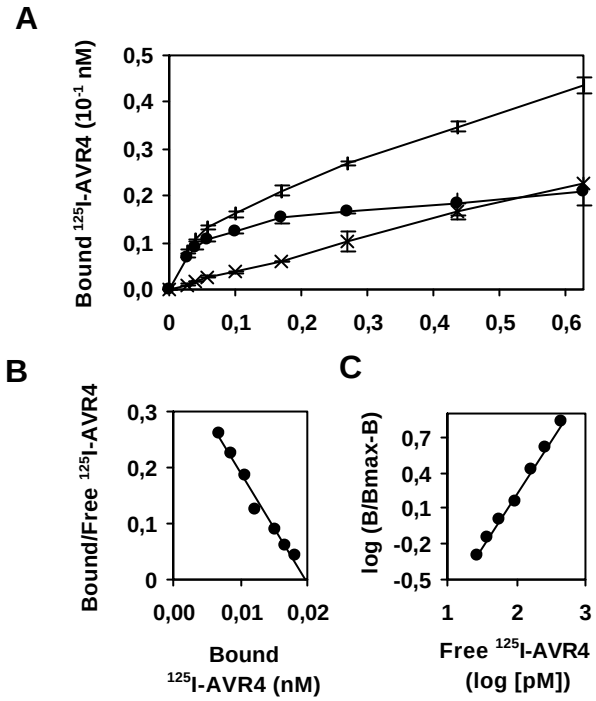

Fig. 3. Saturation of ^{125}I-AVR4 binding to microsomal membranes of MM-Cf4 tomato. A, Microsomal membranes isolated from MM-Cf4 tomato leaves were incubated for 4 h at 37°C with increasing concentrations of ^{125}I-AVR4. Aspecific binding was determined by addition of a 500-fold molar excess of unlabeled AVR4 at *t* = 0 min (x). Specific binding (black dot), which was determined by subtracting aspecific binding (x) from total binding (open diamond), was saturated at a free ligand concentration of about 0.25 nM. Free ligand concentration was calculated by subtracting the initially added ^{125}I-AVR4 from total bound ^{125}I-AVR4. Each data point represents the average of six independent binding assays. **B,** Scatchard plot analysis of data obtained for specific binding of ^{125}I-AVR4 as presented in **A**. From the slope of the Scatchard plot a dissociation constant (K_D = -1/slope) of 0.05 nM was calculated, while extrapolation of the slope to the *x*-axis allowed a calculation of the total receptor concentration (R_t = 0.02 nM = 0.02 pmol ml^{-1} per 48 µg of protein per 0.1 ml of assay) of 0.04 pmol/mg of protein. **C,** Hill plot of data obtained for specific binding (B) of ^{125}I-AVR4 as presented in **A**. B_{max} represents the amount of specific AVR4 binding at saturation level. From the slope of the Hill plot the Hill coefficient (n_H = 0.91) was calculated.

-1/slope) is in the same order of magnitude as the K_D value calculated from the rate constants (Fig. 2C). The total concentration of binding sites (R_t = 0.04 pmol/mg of protein) was determined by extrapolation of the slope to the *x*-axis (Fig. 3B). The calculated Hill coefficient (n_H) of 0.91 is close to 1, suggesting that AVR4 binding to microsomal membranes of MM-Cf4 is non-cooperative (Fig. 3C).

Specificity of ^{125}I-AVR4 binding to microsomal membranes of MM-Cf4

To determine the specificity of AVR4 binding to MFs isolated from MM-Cf4 leaves, a competitive binding assay with AVR4 and AVR9 was performed. Addition of increasing amounts of unlabeled AVR4 protein resulted in progressive inhibition of ^{125}I-AVR4 binding. A 50% inhibition of specific binding (IC_{50}) was observed at an AVR4 concentration of ca. 0.16 nM (Fig. 4). This value is in the same order of magnitude as the K_D values determined in time course experiments and ligand saturation analysis (Figs. 2 and 3). When ^{125}I-AVR4 binding reactions were performed in the presence of concentration up to a 1,000-fold molar excess of unlabeled AVR9, no competition for ^{125}I-AVR4 binding could be detected (Fig. 4). Similarly, other extracellular proteins of *C. fulvum*, ECP1 (Joosten and De Wit, 1988; Van den Ackerveken et al., 1993) (Fig. 4) and ECP4 (Laugé et al. 2000) (data not shown), also did not compete for ^{125}I-AVR4 binding. Together, these data demonstrate that AVR4 binding to microsomal membranes of MM-Cf4 is specific.

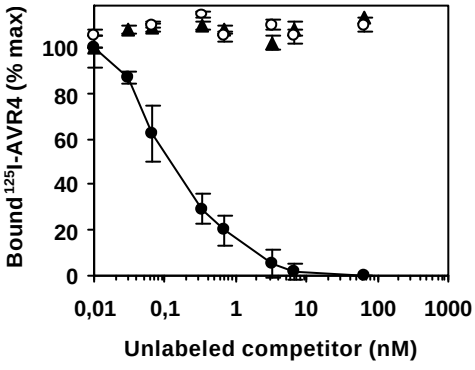

Fig. 4. Competition of ^{125}I-AVR4 binding by unlabeled AVR4, AVR9, or ECP1. Various concentrations of unlabeled AVR4 (black dot), AVR9 (black triangle), and ECP1 (open diamond) were mixed with 0.07 nM ^{125}I-AVR4 and added to microsomal membrane fractions of MM-Cf4 tomato leaves (20 µg of protein per assay). Binding was performed at 37°C for 4 h. Aspecific binding was determined in the presence of a 500-fold molar excess of unlabeled AVR4. Maximal specific binding, determined by subtracting aspecific binding (= 2.2 ± 0.1 pM) from total binding (= 8.7 ± 0.6 pM), was referred to as 100%. Data of six independent binding experiments are presented as the percentage of maximal specific binding.

Binding of ^{125}I-AVR4 to microsomal membranes of MM-Cf0 tomato and nonsolanaceous plant species

We further investigated whether the AVR4 HABS present in MFs isolated from MM-Cf4 leaves, is also present in MM-Cf0 leaves. When binding studies were carried out on MFs of MM-Cf0, a similar AVR4-specific HABS was identified (K_D = 0.08 nM, R_t = 0.057 pmol/mg of protein and n_H = 0.93) (data not shown). In addition, time course experiments were

performed with MFs of MM-Cf0 (Fig. 2), which resulted in similar AVR4-specific binding characteristics as found in MFs of MM-Cf4 plants (data not shown).

To determine whether nonsolanaceous plant species also contain AVR4-specific binding sites, MFs isolated from leaves of *Arabidopsis* spp. and barley grown under regular greenhouse conditions were incubated with ^{125}I-AVR4. Interestingly, these nonsolanaceous plant species showed the presence of an AVR4-specific HABS with K_D values of a similar order of magnitude as found in MM-Cf0 and MM-Cf4 plants (K_D = 0.07 nM and K_D = 0.12 nM for *Arabidopsis* spp. and barley, respectively).

Further biochemical characterization of the AVR4 HABS

To further analyze the nature of the AVR4 HABS, MFs isolated from MM-Cf4 leaves were subjected to a number of different treatments, prior to binding assays with ^{125}I-AVR4. In addition, binding experiments with ^{125}I-AVR9, which served as a control, were carried out (Kooman-Gersmann et al. 1996).

To test for heat-stability of the AVR4 HABS, MFs were incubated at 95°C for 15 min, prior to incubation with ^{125}I-AVR4 or ^{125}I-AVR9. Surprisingly, while heat-treatment completely abolished AVR9 binding, AVR4-specific binding to MFs was reduced by only 15% (Fig. 5A).

In order to assay for proteinase-sensitivity of the AVR4 HABS, MFs were treated with proteinase K. The presence of low concentrations of proteinase K during incubation with ^{125}I-AVR4, however, resulted in breakdown of the AVR4 protein (data not shown). Therefore, proteinase K-treated MFs were heated prior to incubation with ^{125}I-AVR4 in order to inactivate the enzyme. As proteinase K did not affect the stability of AVR9 (Kooman-Gersmann et al. 1996), no heat-treatment was required to inactivate proteinase K prior to ^{125}I-AVR9 binding. While proteinase K-treatment of MFs completely abolished ^{125}I-AVR9-specific binding, no significant effect was found for AVR4 binding (Fig. 5B). Incubation of MFs with heat-treated proteinase K only slightly affected ^{125}I-AVR9-specific binding, whereas the ^{125}I-AVR4-specific binding remained unaffected.

Finally, fractions were isolated from MFs of MM-Cf4 leaves using the two-phase partitioning protocol to enrich for plasma membranes (Larsson et al. 1987). When binding was performed on membranes present in the upper phase, a significant increase in AVR9-specific binding sites, which are localized predominantly in the plasma membrane (Kooman-Gersmann et al. 1996), was found, indicating that plasma membrane-enrichment had occurred (Fig. 5C). In contrast to the increase in the amount of binding sites for AVR9, a significant decrease in AVR4-specific binding sites was found in these plasma membrane-enriched fractions (Fig. 5C). Together, our data indicate that the AVR4 HABS is heat and proteinase K resistant and that it is not associated with the plasma membrane.

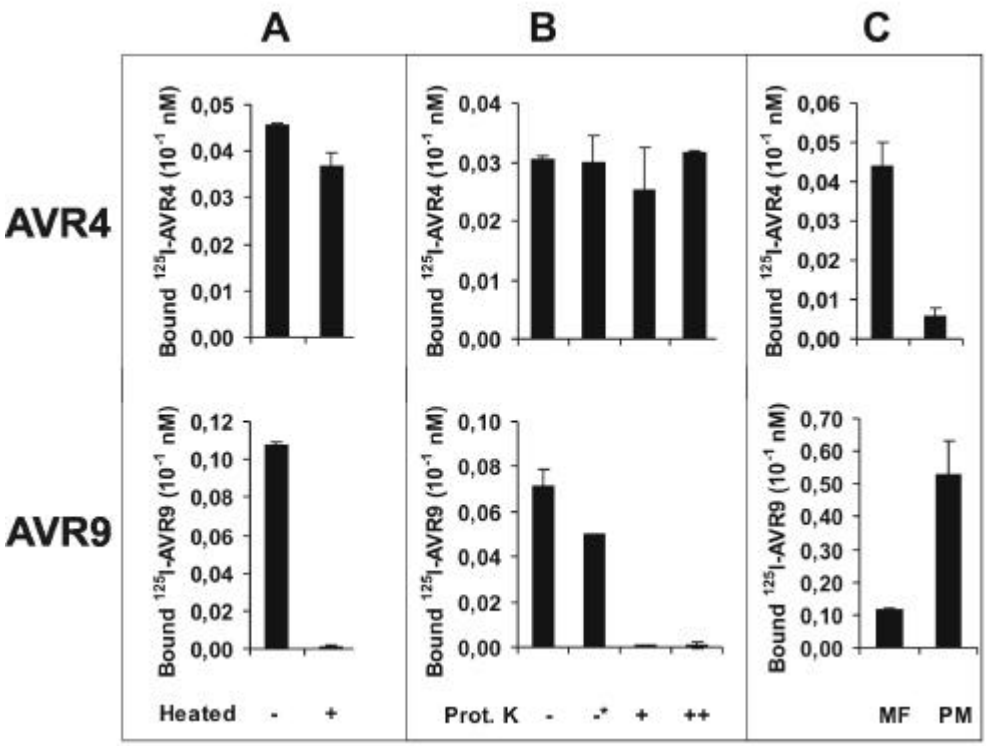

Fig. 5. Biochemical characterization of the AVR4 high affinity-binding site. Heat treatment and proteinase K treatment, respectively, was carried out on microsomal membrane fractions (MFs) isolated from MM-Cf4 tomato leaves. Binding was performed with ^{125}I-AVR4 and ^{125}I-AVR9 at final concentrations of 0.07 nM. Depicted values correspond to specific binding measured 30 min after incubation at 37°C. Each data point represents the average of three binding assays. **A,** Binding of ^{125}I-AVR4 (upper part) and ^{125}I-AVR9 (lower part) to MFs without (-) or with heat treatment (+). **B,** Binding of ^{125}I-AVR4 and ^{125}I-AVR9 to MFs that were not treated (-), treated with inactivated proteinase K (-*) or treated with 5 mg/ml (+) or 15 mg/ml (++) of proteinase K. Prior to incubation with ^{125}I-AVR4, proteinase K-treated MFs were incubated at 95°C for 15 min to inactivate proteinase K. **C,** Binding of ^{125}I-AVR4 and ^{125}I-AVR9 to MFs of MM-Cf4 leaves (MF) and to plasma membrane-enriched fractions isolated from MM-Cf4 leaves (PM).

Inconsistencies in ^{125}I-AVR4-specific binding between different batches of MFs of MM-Cf4 plants

During the assays described above, we observed inconsistencies in ^{125}I-AVR4-specific binding activities between different batches of MFs. In some MFs that were isolated from

leaves of MM-Cf4 plants, as well as from *Arabidopsis* spp., no ^{125}I-AVR4-specific binding sites could be detected. Interestingly, all of these MFs were isolated from plants that were grown in confined compartments. The MFs that exhibited ^{125}I-AVR4-specific binding sites, however, were isolated from MM-Cf4 and *Arabidopsis* plants grown under regular greenhouse conditions.

This observation, together with the predicted nonproteinaceous character of the AVR4 HABS, prompted us to investigate the origin of the AVR4 HABS further. When tomato plants are grown under regular greenhouse conditions, it appears that these plants are often infected with *Oidium lycopersicum*, thereby showing some sporulating colonies on the leaflets. To investigate whether a correlation exists between the presence of fungal contaminants and binding of AVR4 to its HABS, binding assays were performed on MFs isolated from MM-Cf4 leaves that were severely infected with *O. lycopersicum* (i.e., leaves of which 50 to 75% of the surface was infected). Interestingly, the total concentration of binding sites (R_t = 0.09 pmol/mg of protein), which exhibit a K_D value of 0.11 nM, was higher in these MFs (data not shown) compared with the concentration in the MFs isolated from MM-Cf4 plants grown under regular greenhouse conditions (R_t= 0.04 pmol/mg of protein) (Fig. 3).

To discriminate between fungus- and plant-specific binding, as well as fungus-induced, plant-specific binding, we intended to test *O. lycopersicum* for the presence of an AVR4-specific binding site. However, the requirement of plant material for the obligate pathogen *O. lycopersicum* to grow and reproduce hampered the isolation of sufficient fungal

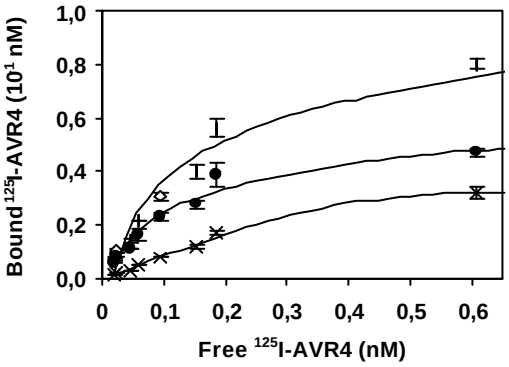

Fig. 6. Saturation of ^{125}I-AVR4 binding to *Cladosporium fulvum* mycelium extracts. *C. fulvum* extracts were incubated with increasing amounts of ^{125}I-AVR4 alone (open diamond) or in the presence of a 500-fold molar excess of unlabeled AVR4 (x). Specific binding (black dot) was determined by subtracting aspecific binding from total binding. Data points are fitted to a hyperbolic curve and each data point represents an average of three independent binding assays.

biomass. Therefore, we performed binding assays on *C. fulvum* mycelium extracts instead. Significantly, an AVR4-specific HABS was detected, which exhibited a K_D value of 0.26 nM, in the absence of plant material (Fig. 6). The total concentration of binding sites in these fractions (R_t = 9.2 pmol/mg of protein) was significantly higher than the R_t in MFs isolated from MM-Cf4 plants (Fig. 3). Moreover, the AVR4 HABS present in the mycelium extracts also appeared to be heat-resistant (data not shown), as demonstrated for the AVR4 HABS present in MFs isolated from MM-Cf4 plants (Fig. 5A). Together, these data demonstrate that the AVR4 HABS is of fungal rather than of plant origin.

Chemical crosslinking of ^{125}I-AVR4 to a distinct compound is dependent on the presence of a fungal infection of tomato

To identify the fungus-derived AVR4 HABS, ^{125}I-AVR4 was chemically crosslinked to *C. fulvum* mycelium extracts and MFs of *O. lycopersicum*-infected MM-Cf4 leaves. ^{125}I-AVR4 was crosslinked to a single band that migrates as a molecule of approximately 85 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in MFs isolated from *O. lycopersicum*-infected MM-Cf4 leaves (Fig. 7A). No AVR4-specific crosslinking was detected in MFs isolated from MM-Cf4 plants grown in confined compartments (Fig. 7B). Significantly, ^{125}I-AVR4 was crosslinked to a band of the same size when binding was performed with *C. fulvum* mycelium extracts (Fig. 7C). In the presence of a 500-fold molar excess of unlabeled AVR4, no crosslinked band could be detected, suggesting that crosslinking to this molecule is specific for AVR4 (Fig. 7A and C). These data demonstrate that the AVR4-specific crosslinked component is of fungal origin rather than of plant origin. Use of glutaraldehyde, *m*-maleimidobenzoyl-*N*-hydrozysuccinimide ester (MBS) and photo-reactive *N*-hydrozysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) as crosslinkers, which all react through different crosslinking mechanisms, gave similar results (data not shown). Presuming that one ^{125}I-AVR4 molecule (molecular mass of 10 kDa) is crosslinked to a single molecule, the predicted molecular weight of the fungus-derived AVR4-specific crosslinked band is approximately 75 kDa.

Discussion

An AVR4-specific high affinity-binding site that is of fungal origin

To characterize the mechanism of specific perception of AVR4 by MM-Cf4 plants, microsomal fractions (MFs) from leaves of such plants were analyzed for the presence of an AVR4-high affinity-binding site (HABS). Although an AVR9-specific HABS was detected in the plasma membranes of MM-Cf9 plants (Kooman-Gersmann et al. 1996), no such binding

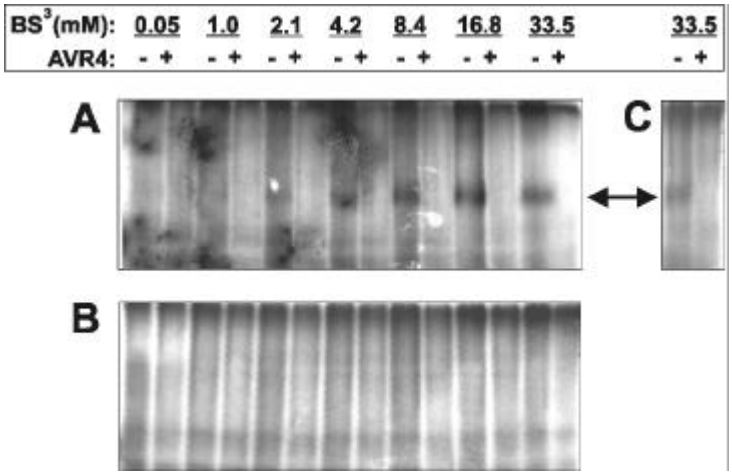

Fig. 7. Chemical crosslinking of ^{125}I-AVR4. The affinity crosslinking experiment was performed on microsomal membrane fractions (MFs) isolated from **A,** *Oidium lycopersicum*-infected MM-Cf4 leaves, **B,** leaves of MM-Cf4 plants that were grown in confined compartments and **C,** on fractions containing *C. fulvum* mycelium extracts. Aliquots of MFs (**A** and **B**, 98 μg of protein per assay) and *C. fulvum* fractions (**C**: 1 μg of protein per assay) were treated with 0.14 nM ^{125}I-AVR4 alone (-) or in combination with a 500-fold molar excess of unlabeled AVR4 (+). Prior to crosslinking, unbound ^{125}I-AVR4 was removed by centrifugation. Crosslinking was initiated by adding different concentrations of the crosslinker bis-sulphosuccinimidyl-suberate (BS^3) (**A** and **B**: final concentrations are indicated) or by adding BS^3 at a final concentration of 33.5 mM (**C**). The crosslinked proteins were separated by SDS-PAGE under reducing conditions and analyzed by autoradiography.

site was found for AVR4. However, an AVR4-specific HABS was found that appeared to originate from fungi present on infected tomato plants, rather than from the tomato plant itself. This fungus-derived AVR4 HABS exhibited all the characteristics expected for ligand-receptor interactions, such as saturability, reversibility and specificity. Furthermore, while the AVR9 HABS has been described as proteinase K and heat sensitive (Kooman-Gersmann et al. 1996), the AVR4-specific HABS proved to be proteinase K and heat resistant, suggesting that the HABS might have a nonproteinaceous nature.

Previously, proteinase- and heat-resistant binding sites have been identified in tobacco plasma membranes for harpin and cryptogein, which are elicitor proteins secreted by *Pseudomonas syringae* pv. *syringae* and *Phytophthora cryptogea*, respectively (Bourque et al. 1999; Lee et al. 2001). Lee and associates (2001) reported that the proteinase-resistant

binding site for harpin is nonproteinaceous. The heat-resistant binding site for cryptogein, however, was shown to be a glycoprotein (Bourque et al. 1999). Indeed, glycosylated proteins repeatedly have been demonstrated to be more resistant against thermal or chemical denaturation and proteolytic degradation compared with their nonglycosylated counterparts (Tanner and Lehle, 1987). Thus, the heat- and proteinase K-resistant fungal AVR4 HABS might represent either a stable glycoprotein or a nonproteinaceous polysaccharide, like glucan or chitin fragments (Bartnicki-Garcia 1968; Kollar et al. 1997). The latter is consistent with high-affinity binding interactions between (plant) proteins and oligosaccharide elicitors that have been identified before (Cosio et al., 1988; Cheong and Hahn, 1991; Shibuya et al., 1993; Baureithel et al., 1994; Asensio et al., 2000; Brandhorst and Klein, 2000; Day et al., 2001; Ito et al., 1997). Interestingly, we identified an AVR4-specific crosslinked molecule of approximately 75 kDa that migrates as a distinct band on SDS-PAGE instead of as a smear, suggesting that it represents a proteinaceous compound rather than a polysaccharide. Significantly, this AVR4-specific molecule of ca. 75 kDa proved to be of fungal rather than of plant origin. Thus, the fungal heat- and proteinase K-resistant AVR4 HABS might represent a molecule of ca. 75 kDa. Alternatively, the fungal AVR4 HABS might represent a polysaccharide. In that case, binding of AVR4 to this polysaccharide might be required for subsequent crosslinking of AVR4 to the molecule of 75 kDa, which in turn exhibits polysaccharide-binding properties.

Our data demonstrate that the AVR4 HABS is present in at least two different classes of fungi (i.e., Fungi Imperfecti and Ascomycetes). Although the exact nature of the HABS is unknown, binding of AVR4 to *C. fulvum* mycelium extracts might indicate that AVR4 in some way contributes to virulence of *C. fulvum* on tomato. It could be possible that AVR4 binds to the surface of *C. fulvum* and protects the fungus against attack by extracellular cell wall-degrading enzymes of tomato. Natural strains that lack a functional AVR4 protein, however, do not exhibit a reduced virulence under laboratory conditions (Joosten et al., 1997). These strains might have compensated for the lack of AVR4 by the production of another secreted protein that protects the fungus, or might indeed exhibit a reduced virulence that is not detected under laboratory conditions. To further elucidate the intrinsic function of AVR4, we are currently identifying the fungal component and components to which AVR4 binds.

The mechanism underlying AVR4 perception by MM-Cf4 plants

Although AVR4 binds to fungal components with high affinity, this interaction is not required for elicitor activity of AVR4 in MM-Cf4 plants, because AVR4 exhibits necrosis-inducing activity when injected into MM-Cf4 leaves in the absence of fungal components. Since growth of *C. fulvum* is restricted to the apoplastic space of tomato, AVR4 perception by MM-

Cf4 plants most likely occurs extracellularly. However, an AVR4-specific binding site in microsomal membranes of MM-Cf4 plants has not yet been detected. This implies that the mechanism of perception of AVR4 by MM-Cf4 plants is different from the perception of several other fungus-derived elicitors, including AVR9, for which a HABS has been found in the plasma membrane of solanaceous plants. The mechanism of AVR4 perception by MM-Cf4 plants remains to be unraveled.

Materials and Methods

Plant materials

Plants were either grown under regular greenhouse conditions or, to obtain pathogen-free plants, in confined compartments. Leaves were harvested from 4-week-old tomato (*Lycopersicon esculentum*) cultivar Moneymaker (MM-Cf0), a near-isogenic line of MM-Cf0 carrying the *Cf-4* resistance gene (MM-Cf4), *Arabidopsis* sp. (*Arabidopsis thaliana* ecotype Columbia) and barley (*Hordeum vulgare* cv. Triumph).

Isolation of microsomal membrane fractions and plasma membranes

Microsomal membrane fractions (MFs) were obtained as described by Sandstrom and associates (1987). Briefly, leaves were homogenized in a blender (Snijders Scientific, Tilburg, The Netherlands) in MB1 buffer (25 mM Tris-HCl, pH 7.5, 250 mM sucrose, 3 mM EDTA, fatty-acid free bovine serum albumin (BSA) at 10 µg/ml, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), following filtration through one layer of cheese cloth and two layers of Miracloth (Calbiochem, La Jolla, CA, U.S.A.) and differential centrifugation steps. Plasma membranes were purified from these microsomal membranes by aqueous two-phase partitioning (Larsson et al. 1987). In brief, three successive extraction steps were performed in a 6.5% polyethylene glycol-6.5% dextran separating system containing 4 mM KCl, 250 mM sucrose, and 5 mM potassium phosphate buffer, pH 7.5. After isolation, the MFs and the plasma membrane-enriched fractions were resuspended in MB3 buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose and 1 mM PMSF) and stored at -80°C. The protein concentration of these fractions was determined using a BCA protein assay reagent with BSA as a standard (Pierce Chemical, Rockford, IL, U.S.A.).

Isolation of *Cladosporium fulvum* mycelium extracts

Cladosporium fulvum race 4 was grown on potato dextrose agar (PDA) or in liquid B5 medium in shake cultures at 22°C and 90 rpm (De Wit and Flach, 1979). *C. fulvum* mycelium extracts were obtained, based on the method as described by Sandstrom and

associates (1987), as follows: liquid B5 medium was inoculated with conidia from ten-day-old PDA cultures of *C. fulvum* (1×10^7 conidia ml^{-1}) and the culture was allowed to grow for 72 h in a rotary shaker at 22°C. The mycelium was harvested and 400 mg of fresh weight was transferred to a 1.5-ml Eppendorf tube. To remove remaining liquid B5 medium, mycelium was pelleted by centrifugation at 9,000 x *g*, resuspended in 500 µl of MB1 buffer, and pelleted again. Subsequently, the mycelium was crushed with a potter. Following centrifugation at 3,000 x *g* for 3 min, the supernatant was transferred to a new Eppendorf tube and centrifuged at 9,000 x *g* for 20 min. The pellet was resuspended in 50 µl of MB3 buffer. These fractions, which are referred to as mycelium extracts, were used in binding assays (0.5 µg of protein per assay) and were used for crosslinking with ^{125}I-AVR4 (1 µg of protein per assay).

DNA manipulation

Standard molecular biological techniques were carried out as described by Sambrook and associates (1989). Polymerase chain reactions (PCRs) were performed with *pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.), according to the manufacturer's instructions. PCR-based cloning was performed with *Avr4* cDNA (Joosten et al., 1997) as a template, to obtain construct pPIC-A4 that allowed production of mature AVR4 by *Pichia pastoris* strain GS115. To introduce a single amino acid change (S78A) at the position of the putative *N*-glycosylation site in the ORF of *Avr4*, a PCR was performed with forward primer cgctcgagaaaagagaggctgaagctaaggccccaa (underlined: *Xho*I site) and reverse primer cgcgaattctacgtatcattgcggcgtctttaccggacacgt**ggc**caggtttgg (underlined: *Eco*RI site, bold: mutation that introduces the Ser-78 to Ala-78 substitution). The amplified PCR product was digested with *Xho*I/*Eco*RI and ligated into the *Xho*I/*Eco*RI-digested expression vector pPIC9 (Invitrogen Corp., Groningen, The Netherlands), resulting in construct pPIC-A4. In this way, the ORF encoding AVR4 was cloned in frame downstream of the á-factor secretion signal, which is fused to the methanol-inducible alcohol oxidase (AOX1) promoter (Ellis et al. 1985). To increase transformation efficiency and to facilitate targeted integration at the His^- locus, resulting in *P. pastoris* His^+ transformants (Cregg and Russell, 1998), pPIC-A4 was linearized with *Sal*I and transformed to *P. pastoris* spheroplasts.

Purification and iodination of AVR4

P. pastoris GS115 His^+/mut^+ transformants transgenic for *Avr4* were selected for production of the AVR4 protein in BMMY medium as described in the Invitrogen manual (version L). Large-scale AVR4 production and purification was performed essentially according to Van den Burg and associates (2001). Briefly, a fermentation protocol was used employing FM22

medium (Stratton et al. 1998), which resulted in high cell densities of *P. pastoris* and high levels of heterologous protein production. Cell-free culture filtrate containing the AVR4 protein was applied to an equilibrated Phenyl Sepharose Fast Flow column (Amersham-Pharmacia, Roosendaal, The Netherlands). Following extensive washing with buffer B (10 mM Tris-HCl, pH 8.6, 1 mM EDTA and 45% saturated $(NH_4)_2SO_4$), the AVR4 protein was eluted with a linear gradient of 300 ml from 100% buffer B to 100% buffer A (10 mM Tris-HCl, pH 8.6 and 1 mM EDTA). The AVR4-containing fractions were pooled, dialyzed against buffer A, and loaded on a Q-Sepharose Fast Flow column (Amersham-Pharmacia). The flow through, which contained the AVR4 protein, was acidified with trifluoroacetic acid (TFA) and injected on a C4 reverse phase-HPLC column (Waters Chromatography Div., Etten-Leur, The Netherlands). Following elution at 30% acetonitril and 0.1% TFA in water, fractions containing AVR4 were collected and lyophilized prior to storage.

The purified AVR4 protein was nonradio-iodinated with ^{127}I and radio-iodinated with ^{125}I following a lactoperoxidase labeling protocol (ANAWA Trading SA, Zürich, Switzerland). AVR9 was iodinated as described by Kooman-Gersmann and associates (1996). Iodination of AVR4 resulted in monolabeled ^{127}I-AVR4 and ^{125}I-AVR4 proteins, as determined by HPLC and MALDI-TOF mass spectrometry (H. A. Van den Burg, unpublished data). These proteins were lyophilized from a solution containing 5% D-mannitol, 0.5% BSA, 1 mM methionine, 21 mM butanesulfonic acid and 80 KIU aprotinin in 225 mM sodium phosphate buffer (pH 5.0). Stock solutions of the proteins were prepared in water and stored at -20°C. The specific radioactivity of mono-iodinated ^{125}I-AVR4 and ^{125}I-AVR9 was 2,130 and 2,200 Ci/mmol, respectively.

Oxidative burst assay

Oxidative burst assays on suspension-cultured tobacco cells expressing *Cf-4* were performed according to De Jong and associates (2000). Briefly, cell suspensions were grown for 4 days after subculturing, at 25°C on a gyratory shaker at 120 rpm. Subsequently, cells were harvested and washed with assay buffer (50 mM MES/NaOH, pH 5.7, 175 mM mannitol, 0.5 mM K_2SO_4, and 0.5 mM $CaCl_2$). Aliquots of the cells (250 mg of cells per 250 µl of assay buffer) were supplied with the fluorescent probe pyranin (10 µg/ml) (Apostol et al. 1989) and cells were challenged with unlabeled AVR4 or ^{127}I-AVR4 at final concentrations of 0.3 nM and 1.5 nM. Directly after addition of the elicitor, the quenching of pyranin fluorescence was recorded at an excitation wavelength of 405 nm and an emission wavelength of 512 nm.

Binding assay

A number of experiments have been carried out to minimize aspecific binding and optimize specific binding. The effects of pH (pH 5.0-pH 9.0), ionic strength (5 mM up to 1 M of $MgCl_2$ or NaCl) and the concentration of MFs (up to 100 μg of protein per assay) were analyzed. MFs were incubated with an appropriate concentration of ^{125}I-AVR4 (up to 0.2 nM final concentration) and unlabeled AVR4 (up to 2,000-fold molar excess) at different temperatures. Eventually, the following experimental conditions were established as optimal. MFs were resuspended in binding buffer (Bb) (10 mM sodium phosphate buffer [NaPi], pH 6.0, 5 mM $MgCl_2$, and 0.1% BSA). Subsequently, aliquots of MFs (48 μg of protein in a final volume of 100 μl) were incubated with ^{125}I-AVR4 or ^{125}I-AVR9 (0.07 nM final concentration) alone (total binding) or in combination with a 500-fold molar excess of unlabeled AVR4 or AVR9 (aspecific binding), respectively. The same conditions were used when binding was performed with ^{125}I-AVR4 or ^{125}I-AVR9 on plasma membrane-enriched fractions (48 μg of protein per reaction). In competition experiments, aliquots of MFs (20 μg of protein per assay) were incubated with ^{125}I-AVR4 and various concentrations of different competitor proteins. Specific binding was determined by subtracting aspecific binding from total binding. Binding assays were terminated by filtration through glass fibre filters (Multiscreen-FB, 1.0 μm glass fibre, Millipore Corp., Etten-Leur, The Netherlands) that were impregnated with 0.5% (wt/vol) polyethylenimine for 30 min and subsequently washed with 100 μl of wash buffer (Bb supplied with 1 M NaCl, pH 6.0) (Bruns et al. 1983). Following filtration, filters were washed three times with 100 μl of wash buffer. The radioactivity retained on the filters was measured by liquid scintillation counting (model LS-6000 TA; Beckman Instruments, Irvine, CA, U.S.A.)

Proteinase treatment

MFs (48 μg of protein/reaction) were incubated in MB3 buffer for 2 h at 25°C with proteinase K from *Tritirachium album* (Merck) at 5 or 15 mg/ml. In control assays, MFs were incubated with 15 mg/ml of heat-treated proteinase K (incubated for 15 min at 100°C) at similar conditions. Prior to incubation with ^{125}I-AVR4, the mixture containing the microsomal membranes and proteinase K was heated for 15 min at 95°C.

Chemical crosslinking of ^{125}I-AVR4

MFs (48 μg per reaction) were incubated with ^{125}I-AVR4 at a final concentration of 0.07 nM, either alone or in combination with a 500-fold molar excess of unlabeled AVR4. Following incubation with ^{125}I-AVR4 for 4 h at 37°C, the incubation mixture was centrifuged for 30 min at 100,000 x *g* to remove unbound ^{125}I-AVR4. The pellet was resuspended in 1 mM NaPi

buffer, pH 6.0 and crosslinking was initiated by adding increasing amounts (0.05 mM up to 33.5 mM final concentration) of the crosslinking reagents bis-sulphosuccinimidyl-suberate (BS^3), MBS, sulfo-HSAB (Pierce Chemical, Rockford, IL, U.S.A.), or glutaraldehyde (Sigma, Zwijndrecht, The Netherlands). Crosslinking reactions were performed at 25°C for 30 min and reaction mixtures containing sulfo-HSAB were subsequently irradiated for 5 min with UV light (254 nm). All crosslinking reactions were terminated by adding SDS loading buffer (50 mM Tris-HCl, pH 6.8, 5% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% 2-mercaptoethanol and 0.0025% bromophenol blue). Samples were incubated at 95°C for 5 min and separated by SDS-PAGE on gels containing 7.5% (wt/vol) acrylamide (Laemmli 1970). Gels were dried and analyzed by autoradiography.

Acknowledgments

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Chapter 4

The tomato pathogen *Cladosporium fulvum* evades *Cf-4*-mediated resistance by secreting unstable disulfide bond AVR4 mutants that are still capable of binding to chitin

This chapter will be submitted for publication with minor modifications by Harrold A. van den Burg, Nienke Westerink, Kees-Jan Francoijs, Ronelle Roth, Esmeralda Woestenenk, Sjef Boeren, Pierre J.G.M. de Wit, Matthieu H.A.J. Joosten, and Jacques Vervoort.

The tomato pathogen *Cladosporium fulvum* evades *Cf-4*-mediated resistance by secreting unstable disulfide bond AVR4 mutants that are still capable of binding to chitin

Abstract

Disease resistance in plants commonly requires two complementary genes, an avirulence gene in the pathogen and a matching resistance gene in the host. The AVR4 elicitor protein of the tomato pathogen *Cladosporium fulvum* induces defense responses in tomato carrying the *Cf-4* resistance gene. AVR4 contains 8 cysteine residues, all of which are involved in disulfide bonds. Here, we elucidated the disulfide pattern of AVR4 and its role in conformational stability and elicitor activity. Using a method known as partial reduction/mass mapping, the disulfide bonds present in AVR4 were identified as Cys^{11}-Cys^{41}, Cys^{21}-Cys^{27}, Cys^{35}-Cys^{80}, and Cys^{57}-Cys^{72}. A motif-based search based on the disulfide bond pattern and the spacing of the cysteine residues of AVR4 revealed that AVR4 contains an invertebrate chitin-binding domain (inv ChBD). Three of the four disulfide bonds present in AVR4, i.e. [11-41], [35-80], and [57-72], are conserved in inv ChBDs and moreover, these three bonds appear to be required for conformational stability of AVR4. Independent disruption of each of these three disulfide bonds in AVR4 resulted in a protein that is sensitive to proteolysis. The Cys-to-Tyr mutations identified in natural strains of *C. fulvum* involve two of these three conserved disulfide bonds, i.e. [11-41] and [35-80]. Moreover, AVR4 mutant proteins with a disruption in one of these disulfide bonds are still capable of binding to chitin. When bound to chitin, these disulfide bond AVR4 mutants are less sensitive to proteolytic breakdown. Thus, while evasion of *Cf-4*-mediated resistance by *C. fulvum* appears to result from instability and protease sensitivity of AVR4 mutant proteins present in the apoplastic space of tomato, these unstable AVR4 mutants are still capable of binding to chitin *in vitro*, implying that they preserve their putative intrinsic function in protecting the cell wall of *C. fulvum* against plant chitinases is retained.

Introduction

Gene-for-gene-based disease resistance in plants commonly requires two complementary genes, an avirulence (*Avr*) gene in the pathogen and a matching resistance (*R*) gene in the host (Flor, 1971; Dangl and Jones, 2001). The *Cf* resistance genes of tomato mediate

specific recognition of extracellular elicitor proteins encoded by *Avr* genes of the pathogenic fungus *Cladosporium fulvum* (Joosten and De Wit, 1999). The *Avr* genes of *C. fulvum* and their matching *Cf* genes have become valuable instruments to investigate signal transduction pathways leading to plant disease resistance (Romeis et al., 1999 and 2001b; Durrant et al., 2000; De Jong et al., 2000 and 2002a; Rivas et al., 2002a). To obtain sustainable resistance, the *Cf* resistance genes were introgressed from wild *Lycopersicon* species into commercial tomato cultivars. However, due to selection pressure, new strains of *C. fulvum* emerged that had developed the ability to overcome these introgressed resistance traits by modification of the *Avr* gene products (Day, 1957). While some *Avr* genes in these virulent *C. fulvum* strains were found to be absent (Van Kan et al., 1991), others were found to contain point mutations or transposon insertions (Luderer et al., 2002b). It appeared, however, that natural strains of *C. fulvum* carrying these mutated *Avr* genes do not exhibit reduced virulence under laboratory conditions (Van Kan et al., 1991; Joosten et al., 1994; Joosten et al., 1997; Luderer et al., 2002b), suggesting that AVR proteins are not essential for virulence or that the modified AVR isoforms still contribute to virulence of *C. fulvum*. The genetic variation observed so far, is strictly limited to race-specific *Avr* genes and is absent in genes that encode other extracellular elicitor proteins (ECPs) of *C. fulvum* (Luderer et al., 2002a).

Although the intrinsic function of AVR proteins of *C. fulvum* during infection remains to be elucidated, AVRs of other pathogens are reported to contribute to virulence by suppressing plant defense responses (Kjemtrup et al., 2000; White et al., 2000; Bonas and Lahaye, 2002). This implies that modification of the AVRs, in order to evade induction of plant defense responses, might carry a virulence penalty for the pathogen.

For AVR4 of *C. fulvum*, a role in virulence has been proposed, as it binds specifically to chitin *in vitro* as well as to fungal cell walls *in planta* (Van den Burg et al., 2003). Moreover, AVR4 has been demonstrated to protect fungi against plant chitinases (Van den Burg et al., 2003). Previously, it has been demonstrated that all strains of *C. fulvum*, except one, evade *Cf-4*-mediated resistance by secreting AVR4 mutant proteins that carry single amino acid substitutions (Joosten et al., 1994 and 1997). The point mutations involve (i) a deletion of a single nucleotide, resulting in a truncated AVR4 protein, (ii) mutations that replace Thr^{66} by Ile and Tyr^{67} by His, and (iii) mutations that result in single Cys-to-Tyr substitutions at positions 64, 70, and 109. The latter residues correspond to Thr^{36}, Tyr^{37}, Cys^{35}, Cys^{41}, and Cys^{80} of the mature AVR4 protein, respectively (Joosten et al., 1997). Interestingly, some of these AVR4 mutant proteins still exhibited necrosis-inducing activity (NIA), when transiently expressed in tomato using potato virus X (PVX) (Joosten et al., 1997). However, none of these AVR4 isoforms could be detected in apoplastic fluid isolated from tomato leaves that

were inoculated with strains of *C. fulvum* that produce the respective AVR4 mutant proteins (Joosten et al., 1997). Here, we examined the chemical and biological properties of native and mutant AVR4 proteins secreted by strains of *C. fulvum*, with special emphasis on the role of cysteine residues.

Mass spectrometry revealed that all eight Cys residues in native AVR4 are involved in disulfide bonding (Van den Burg et al., 2001). The disulfide bond pattern and the sequential spacing of the Cys residues were found to determine the folding of most small, secreted proteins (Harrison and Sternberg, 1996; Mas et al., 1998). Here, we elucidated the disulfide pattern of AVR4 and its role in conformational stability and elicitor activity. Using a method known as partial reduction/mass mapping, the disulfide bonds of AVR4 were identified as Cys^{11}-Cys^{41}, Cys^{21}-Cys^{27}, Cys^{35}-Cys^{80}, and Cys^{57}-Cys^{72}. A motif search, based on the disulfide bond pattern and the spacing of the cysteine residues of AVR4, revealed that AVR4 contains an invertebrate (inv) chitin-binding domain (ChBD) (Shen and Jacobs-Lorena, 1999). Here, we show that three of the four disulfide bonds present in AVR4, excluding [21-27], are conserved in the inv ChBD. Independent disruption of each of these three disulfide bonds in AVR4 resulted in a protein that is sensitive to proteolysis, indicating that these three disulfide bonds are required for conformational stability of AVR4. The Cys-to-Tyr mutations identified in natural strains of *C. fulvum* involve two of these three conserved disulfide bonds, i.e. [11-41] and [35-80]. Moreover, AVR4 mutant proteins with a disruption in one of these disulfide bonds are still capable of binding to chitin *in vitro*. When bound to chitin these AVR4 mutant proteins are less sensitive to proteolytic breakdown. Thus, while triggering of *Cf-4*-mediated defense responses is evaded by production of AVR4 mutant proteins that are degraded by proteases present in the apoplast of tomato, these AVR4 mutants retained the ability to bind to chitin, which implies that they preserve their putative intrinsic function in protecting *C. fulvum* against plant chitinases.

Results

Four Cys residues of AVR4 are essential for induction of *Cf-4*-mediated defense responses in tomato

By using PVX-mediated expression in *Cf-4* tomato, it has previously been demonstrated that AVR4 mutant proteins carrying Cys-to-Tyr substitutions at positions 35 and 80 exhibited reduced necrosis-inducing activity (NIA) as compared to native AVR4, whereas AVR4 mutant carrying a Cys^{41}Tyr substitution did not show any NIA (Joosten et al., 1997). To determine whether other Cys residues, for which no mutations were found in natural strains of *C. fulvum*, are also required for NIA of AVR4, we replaced all individual Cys residues

independently by Ala. The mutant *Avr4* constructs were subsequently introduced into the PVX vector and inoculated onto four-week-old *Cf-4* tomato plants. As found for AVR4 mutant carrying $Cys^{41}Tyr$ substitution, it appeared that mutant proteins carrying an Ala substitution at positions Cys^{11} or Cys^{41} did not exhibit any NIA (Fig. 1). Moreover, inoculation of PVX::*Avr4* constructs encoding AVR4 mutant proteins carrying a $Cys^{21}Ala$ or $Cys^{27}Ala$ substitution resulted in the same NIA as demonstrated for constructs that encode AVR4 mutant proteins carrying $Cys^{35}Ala$ and $Cys^{80}Ala$ substitutions. The latter two mutants are as active as the $Cys^{35}Tyr$ and $Cys^{80}Tyr$ substitution mutants found in natural strains of *C. fulvum.* Thus, the finding that AVR4 mutant proteins carrying Cys-to-Ala substitutions at positions 11, 41, 57, and 72 all show no NIA suggests that these residues are involved in the formation of disulfide bonds in AVR4. As these mutants already lack NIA, subsequent analysis using double Cys-to-Ala substitution mutants would not result in further assignment of disulfide bond pattern of AVR4 involving these Cys residues. For residues Cys^{21}, Cys^{27}, Cys^{35} and Cys^{80} of which the single Cys-to-Ala substitution mutants showed reduced NIA, however, six additional PVX::*Avr4* constructs encoding AVR4 mutant proteins with double Cys-to-Ala substitutions were obtained. Four of the six double mutants did no longer exhibit NIA, whereas mutants carrying $Cys^{21,27}Ala$ and $Cys^{35,80}Ala$ substitutions were as active as mutants carrying one of the corresponding single Cys-to-Ala substitution (Table 1). These data suggest that Cys^{21} is connected with Cys^{27} and Cys^{35} with Cys^{80}.

Fig. 1. Necrosis-inducing activity of PVX*::Avr4* derivatives in MM-Cf4 tomato plants. The first group of four-week-old MM-Cf4 tomato plants were inoculated with PVX::*Avr4* derivatives containing a mutation in either Cys^{11} (1), Cys^{41} (5), Cys^{57} (6), or Cys^{72} (7). Note that these plants exhibited no systemic necrosis, but rather systemic mosaic symptoms induced by the viral infection. The second group of MM-Cf4 plants was inoculated with PVX::*Avr4* derivatives containing a mutation in Cys^{21} (2), Cys^{27} (3), Cys^{35} (4), or Cys^{80} (8). Note that these plants developed severe necrotic symptoms. The photograph was taken 10 days post inoculation. The Cys residues of AVR4, for which modifications were found in strains of *C. fulvum* virulent on plants carrying *Cf-4*, are underlined.

Table 1. Necrosis-inducing activity (NIA) of PVX::*Avr4* derivatives on MM-Cf4 tomato plants.

Single Cys-to-Ala substitution	NIA	Double Cys-to-Ala substitutions	NIA
-	+++++	$Cys^{21,27}Ala$	++
$Cys^{11}Ala$	-	$Cys^{21,35}Ala$	-
$Cys^{21}Ala$	++	$Cys^{21,80}Ala$	-
$Cys^{27}Ala$	++	$Cys^{27,35}Ala$	-
$Cys^{35}Ala$	++	$Cys^{27,80}Ala$	-
$Cys^{41}Ala$	-	$Cys^{35,80}Ala$	++
$Cys^{57}Ala$	-		
$Cys^{72}Ala$	-		
$Cys^{80}Ala$	++		

Chemical reduction and cyanylation of the disulfide bonds in AVR4

In order to determine the disulfide bond pattern of AVR4, we followed a chemical approach. We partially reduced the disulfide bonds by treating AVR4 with tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) at pH 3.0. This treatment minimized intramolecular rearrangements of disulfide bonds (Gray, 1993; Van den Hooven et al., 2001). The free cysteine thiol groups were directly modified by alkylation with 1-cyano-4-diethylamino-pyridinium (CDAP) under acidic conditions and the resulting peptides were separated by reverse-phase HPLC (Fig. 2). In the presence of a 6-fold molar excess of TCEP, approximately 50% of native AVR4 was reduced as observed by an increased HPLC retention time of the formed species (Fig. 2). Subsequent MALDI-TOF mass spectrometry (MS) identified four peaks that represent AVR4 species with one reduced disulfide bond (indicated by *, Fig. 2). Peaks that elute at higher acetonitrile concentrations contain AVR4 species with more than one disulfide bond reduced, which is consistent with the finding that higher retention times reflect an increase in hydrophobicity and therefore an increase in the unfolded state of the AVR4 species (data not shown). The peaks eluting at 30.8 min and 30.9 min could not be separated in one run on HPLC, but after an additional run both species appeared more than 85% pure.

Assignment of the disulfide bonds in AVR4 using mass spectrometry

To determine which of disulfide bond was reduced in the AVR4 *des*-species, the collected protein fractions were lyophilized and dissolved in 1 M NH_4OH to induce base-catalyzed cleavage of the peptide bond that precedes the two modified half-cystines. This cleavage leads to peptide iminothiazolidine derivatives (*tz*) (Wu and Watson, 1998). The double cleavage reaction is not always complete and as a side-reaction β-elimination might occur at

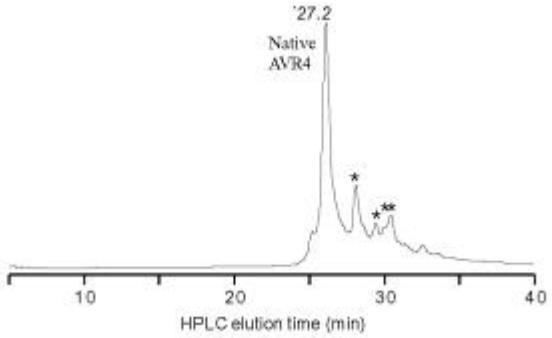

Fig. 2. HPLC profile of the partially reduced and cyanylated AVR4 protein mixture. The peaks marked with (*) correspond to four single reduced and cyanylated AVR4 species (*des*-species), as deduced by mass spectrometry. Each reaction involved 100 µg of AVR4 that reacted with a 6-fold molar excess of TCEP. The relative abundance of native AVR4 was 48% (with a retention time [t_r] of 27.2 min) and for the AVR4 *des*-species: 17% (t_r = 28.4 min), 6.1% (t_r = 29.9 min), 4.3% (t_r = 30.8 min) and 8.2% (t_r = 30.9 min).

one of the half-cystines, thereby preventing cleavage at that site (Degani and Patchornik, 1974; Wu and Watson, 1997).

The peptide fragments with the three remaining disulfide bonds were completely reduced to release the individual peptide fragments. Thus, for each *des*-species, the final reaction mixture yields a maximum of five peptide fragments, i.e. three peptide fragments originating from the complete double cleavage reaction and two fragments originating from the single cleavage reaction (due to β-elimination). The masses of the peptide fragments were subsequently analyzed by MALDI-TOF MS (Fig. 3).

Assignment of the disulfide bonds was performed in a two-step approach. First, the mass peaks that correspond to peptide fragments from the N- and C-terminus up to the first reduced half-cystine were assigned. In Fig. 3A, for example, the mass peaks *m/z* 2286.6 Da and 6673.9 Da correspond to the peptide fragments encompassing the N-terminal residues [1-20] and the C-terminal residues [27-86], where Cys^{27} is converted into an *itz*-derivative (Table 2). This assignment was confirmed by the presence of additional mass peaks, i.e. *m/z* 2882.3 Da and 7271.3 Da, which both originated from the β-elimination reaction (i.e. the peptide fragments [1-26] with a β-elimination at Cys^{21} and [21-86] with a β-elimination at Cys^{27}, respectively). The remaining fragment (*itz* 21-26) was too small to be detected due to the setting of the lower mass limit (of the linear mode) at 1000 Da. Thus, the MS spectrum shown in Fig. 3A revealed that a disulfide bond connects Cys^{21} and Cys^{27}. Comparable

mass peak analyses of the peptide mixtures generated from the other three AVR4 *des*-species resulted in the assignment of disulfide bonds [11-41], [57-72] and [35-80] (Fig. 3B, 3C and 3D, respectively). Together, these data demonstrate that a disulfide bond connects Cys^{21} and Cys^{27}, as well as Cys^{35} and Cys^{80}, which is consistent with the data obtained in the PVX bioassays (Fig. 1; Table 1). Moreover, the mass mapping allowed us to assign two additional disulfide bonds, [11-41] and [57-72], in AVR4.

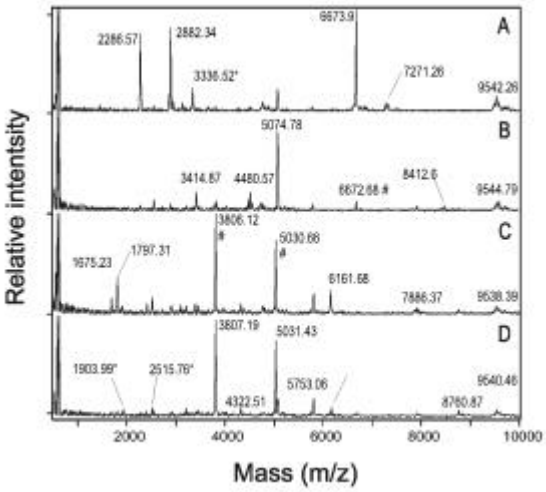

Fig. 3. MALDI-TOF mass spectra of the four peptide mixtures obtained after base-induced cleavage of the peptide bond and full reduction of the AVR4 *des*-species. The mass per charge (m/z) is given for those peaks that are used to assign what Cys residues are involved in formation of intramolecular disulfide bonds. The panels **A-D** represent (in order of elution from the HPLC) peptide mixtures containing AVR4 *des*-species **A,** [21-27], **B,** [11-41], **C,** [57-72] and **D,** [35-80], respectively. The mass peaks that correspond to double charged mass peaks ($[M + 2H]^{2+}$) (*) are indicated. Re-occurring mass peaks (#) reflect cross-contamination with other AVR4 *des*-species.

Three disulfide bonds in AVR4 are required for its conformational stability

Previously, no AVR4 mutant proteins could be detected in apoplastic fluid (AF) isolated from tomato plants infected by strains of *C. fulvum* producing these isoforms. Therefore, we compared the stability of the four mutant AVR4 *des*-species with native AVR4. It appeared that, in the absence of AF, native AVR4 as well as the AVR4 *des*-species are stable over the time span of one hr (Fig. 4). However, when mutant AVR4 *des*-species were incubated with

Table 2. Theoretical masses (m/z) of peptide fragments obtained after base-catalyzed cleavage of peptide bonds in AVR4 *des*-species*.

Des-[11-41]		*Des*-[21-27]		*Des*-[35-80]		*Des*-[57-72]	
Fragment	m/z	Fragment	m/z	Fragment	m/z	Fragment	m/z
1-10	1144.3	1-20	2285.6	1-34	3805.4	1-56	6160.2
itz 11-40	3415.9	*itz* 21-26	676.8	*itz* 35-79	5031.8	*itz* 57-71	1800.0
itz 41-86	5075.9	*itz* 27-86	6673.7	*itz* 80-86	798.9	*itz* 72-86	1675.9
β-(1-40)	4482.2	β-(1-26)	2884.4	β-(1-79)	8759.2	β-(1-71)	7882.2
β-(11-86)	8413.8	β-(21-86)	7272.4	β-(35-86)	5752.7	β-(57-86)	3397.9

* ; AVR4 *des*-species represent cyanylated AVR4 proteins that carry one reduced disulfide bond (Fig. 2). Base-catalyzed cleavage of the peptide bond that precedes the two modified half-cystines results in peptide iminothiazolidine derivatives (*itz*). A side-reaction occurs when the double cleavage reaction is not complete, resulting in β-elimination at one of the half-cystines (β). For the majority of the peptide fragments, the relative deviation between measured and calculated masses is less than 0.05%.

AF, three of the four species could no longer be detected, whereas native AVR4 and [21-27]-AVR4 *des*-species were not affected. Moreover, in the presence of protease inhibitors (Fig. 4, +AF, + Prot. Inh.), treatment with AF did no longer affect the stability of these AVR4 *des*-species, suggesting that these AVR4 species are degraded by proteases present in AF. Thus, unlike disulfide bond [21-27], disulfide bonds [11-41], [35-80] and [57-72] are required for conformational stability of AVR4.

AVR4 contains an invertebrate chitin-binding domain

The identified disulfide bond pattern of AVR4 was further used to perform a motif-based search (URL:http://motif.genome.ad.jp). This search identified a sequence stretch in AVR4 that was homologous to a stretch present in proteins of the invertebrates *Manduca sexta, Brugia* malayi, and *Penaeus japonicus*, all of which exhibit chitinase activity (Shen and Jacobs-Lorena, 1999). The homology, however, is restricted to the C-terminal domain, which contains six conserved cysteine residues. Moreover, this domain has recently been identified as the invertebrate chitin-binding domain (inv ChBD) (Shen and Jacobs-Lorena, 1999). For one member of the inv ChBD family, tachycitin of Japanese Horseshoe crab (*Tachypleus tridentatus*), the disulfide bond pattern has been solved (Kawabata et al., 1996). Unlike AVR4, tachycitin contains five disulfide bonds of which three are conserved within the inv ChBD. Subsequent sequence alignment revealed that the disulfide bonds [11-41], [35-80], and [57-72] present in AVR4 are conserved within the ChBD family (Fig. 5). The additional disulfide bond [21-27] within AVR4 does not share homology with disulfide bonds

present in tachycitin. Opposed to AVR4 and tachycitin, the other members of the inv ChBD family do not contain additional cysteine residues (Fig. 5). A three-dimensional model of AVR4 was constructed using the 3D-structure of tachycitin as a template (Suetake et al., 2000) (data not shown). The modeled structure of AVR4 contains the secondary structural elements as found in tachycitin, but the sequence insertion encompassing the disulfide bridge [21-27] was too large to construct a reliable model for that part of the protein.

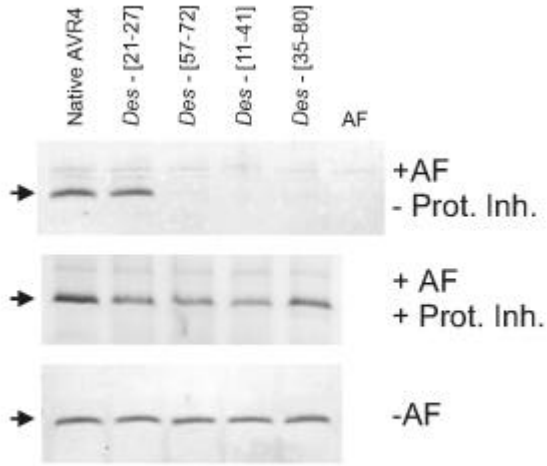

Fig. 4. Stability of disulfide bond AVR4 mutants in the presence of apoplastic fluid. Protein samples were incubated for 1 hr at 30°C in the absence (-AF) or presence (+AF) of apoplastic fluid (AF). As a control, AF was supplied with protease inhibitors (+Prot. Inh.). The stability of the AVR4 (mutant) proteins was analyzed by SDS-PAGE. Samples were loaded in the same order as they eluted from the RP-HPLC column (Fig. 2).

Disulfide bond mutants of AVR4 are still capable of binding to chitin

In a concurrent paper, we have demonstrated that native AVR4 specifically binds to chitin but not to other cell wall polysaccharides (Van den Burg et al., 2003). Moreover, for human chitinase, it has been shown that six conserved Cys residues that belong to the inv ChBD are required for its chitin-binding activity (Tjoelker et al., 2000). To test whether the AVR4 *des*-species still exhibit chitin-binding activity, we incubated these proteins with chitin for 1 hr. Following pelleting of chitin by centrifugation and subsequent analysis of the pellet and supernatant fractions by SDS-PAGE, all four AVR4 *des*-species were detected in the pellet fraction (Fig. 6). This suggests that absence of one disulfide bridge in AVR4 does not abolish its ability to bind to chitin *in vitro*. However, for [57-72] AVR4 *des*-protein a higher level of AVR4 protein remained in the supernatant fraction, suggesting a decreased affinity of this protein for chitin (Fig. 6). However, after a prolonged incubation with chitin (1 to 4 hrs) all of the [57-72] AVR4 *des*-protein was found in the pellet fraction (data not shown). Thus, in spite of the fact that differences in affinity of [57-72] AVR4 *des*-protein for chitin have been observed, our data demonstrate that disruption of one disulfide bond does not abolish the

chitin-binding activity of AVR4. It should be mentioned here that [57-72] AVR4 *des*-protein might have been contaminated with [35-80] AVR4 des-protein, which could in turn have interfered with the chitin-binding assay. This implies that, as the [35-80] AVR4 des-protein exhibits the same affinity for chitin as native AVR4, the [57-72] AVR4 des-protein might even exhibit a lower affinity for chitin than observed in our assay.

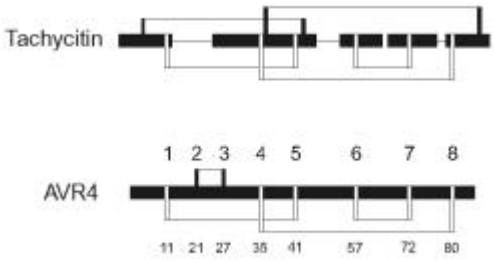

Fig. 5. Schematic diagram of the disulfide bond pattern of tachycitin and AVR4. The connected open bars represent three conserved disulfide bonds of the invertebrate chitin-binding domain (inv ChBD). The connected filled bars represent additional disulfide bonds. Gaps in the alignment are indicated by a thin-line in tachycitin. The residue numbers (bottom) and the successive order (top) of the Cys residues of AVR4 are depicted. The diagram is based on the disulfide bond pattern of tachycitin and the spacing of cysteine residues of other inv ChBDs, using the consensus found in SMART and PFAM protein domain databases (ID codes SM00494 / PF01607) (Schultz et al., 1998; Bateman et al., 2002; Van den Burg et al., 2003).

Binding to chitin protects disulfide bond mutants of AVR4 against proteolytic breakdown

During growth in tomato, *C. fulvum* remains confined to the intercellular spaces of tomato leaves (De Wit, 1977). It has been proposed that after secretion AVR4 binds directly to regions of the cell wall of hyphae of *C. fulvum,* where chitin is accessible (Van den Burg et al., 2003). When the exposed chitin is saturated with AVR4, free AVR4 is thought to be released into the apoplast. We investigated whether the AVR4 *des*-species were less sensitive to proteolytic degradation after binding to chitin. Therefore, we pre-incubated these AVR4 *des*-species with chitin for four hrs, rather than one hr, to ensure a complete association between these proteins and chitin. After incubation with chitin, the solutions were supplied with AF and stability of AVR4 mutant proteins was followed in time. Over the period of four hrs, native AVR4 and the [21-27] AVR4 *des*-protein that were incubated with chitin, remained fully stable in the presence of AF (Fig. 7; Table 3). Moreover, when bound to chitin, normally unstable AVR4 *des*-species [11-41], [35-80] and [57-72] are less sensitive to degradation by proteases present in AF.

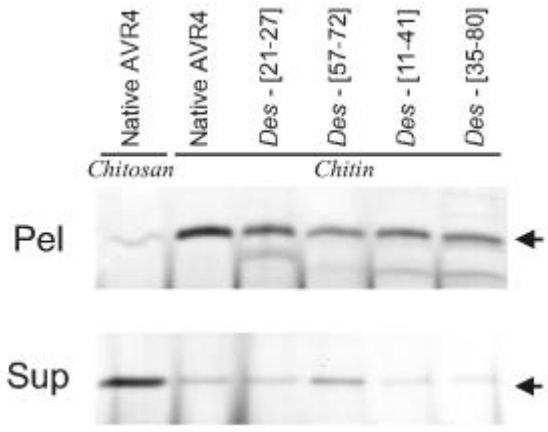

Fig. 6. Disulfide bond mutants of AVR4 are still capable of binding to chitin. SDS-PAGE was used to assay the amount of AVR4-*des* protein that remained in solution (Sup) and the amount that bound to chitin in the pellet (Pel). Chitosan, to which native AVR4 does not bind, was included as a negative control. Note that more [57-72] AVR4 *des*-protein remains in solution as compared to the other AVR4 *des*-proteins.

Discussion

Here, we elucidated the disulfide bond pattern of AVR4 by a method known as partial reduction/mass mapping. This method allowed us to selectively disrupt a single disulfide bond in AVR4 and to purify and isolate these so-called AVR4 *des*-species for further research. The following Cys residues in AVR4 are connected: Cys^{11}-Cys^{41}, Cys^{21}-Cys^{27}, Cys^{35}-Cys^{80} and Cys^{57}-Cys^{72}. A motif search based on the disulfide bond pattern and the spacing of the cysteine residues of AVR4 identified the presence of an invertebrate chitin-binding domain (inv ChBD) in AVR4 (Shen and Jacobs-Lorena, 1999). Moreover, structural homology was found between the disulfide bond pattern of AVR4 and that of tachycitin, a member of the inv ChBD family (Kawabata et al., 1996). For the other members of the inv ChBD family no disulfide bonding has thus far been elucidated.

For AVR4, we demonstrated that three disulfide bonds, excluding Cys^{21}-Cys^{27}, are essential for protein stability and correspond to the six Cys residues that are conserved amongst proteins of the ChBD family (Shen and Jacobs-Lorena, 1999). Although for human chitinase it has been demonstrated that all six Cys residues are essential for chitin

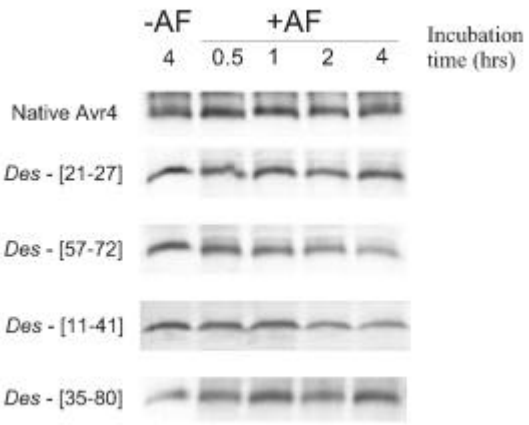

Fig. 7. Binding to chitin protects disulfide bond mutants of AVR4 against proteolytic breakdown. To test whether binding to chitin protects normally unstable disulfide bond mutants of AVR4 against proteases present in apoplastic fluid (AF) of tomato, AVR4 *des*-species were pre-incubated with chitin, followed by an incubation at 30°C for 4 hrs in the absence (-) or the presence (+) of AF. The stability of the AVR4 *des*-species was analyzed by SDS-PAGE.

recognition (Tjoelker et al., 2000), for AVR4 disruption of one of the three disulfide bonds does not abolish its ability to bind to chitin *in vitro*. It has been demonstrated that strains of *C. fulvum* evade *Cf-4*-mediated resistance by modification of the *Avr4* gene product. Most modifications involved substitution of Cys residues at positions 35, 41 and 80 by Tyr (Joosten et al., 1997), indicating that disruption of only two of the three conserved disulfide bonds have, so far, contributed to evasion of AVR4 recognition. When AVR4 mutant proteins carrying a disruption in one of these conserved disulfide bonds were exposed to apoplastic fluid (AF) isolated from healthy or *C. fulvum*-infected tomato leaves, the proteins are rapidly degraded as a result of protease activity present in AF. Thus, as previously suggested by Joosten et al. (1997), *C. fulvum* evades *Cf-4*-mediated resistance by production of AVR4 isoforms that are degraded by proteases present in the apoplast of tomato leaves. Interestingly, these unstable AVR4 isoforms still exhibit chitin-binding activity. However, disruption of disulfide bond [57-72] was found to reduce the affinity of AVR4 for chitin. Moreover, the 3D-structure of tachycitin shows that disulfide bond [57-72] is present in a functional chitin-binding moiety (Suetake et al., 2000), which supports a decreased affinity for chitin when this bond is disrupted. Assuming that binding of AVR4 to chitin protects *C. fulvum* against plant chitinases, as proposed by Van den Burg et al. (2003), strains of *C. fulvum* that produce AVR4 mutant proteins lacking disulfide bond [57-72] to evade *Cf-4*-mediated resistance would probably be more sensitive towards plant chitinases.

Table 3. Overview of necrosis-inducing activity, stability and chitin-binding ability of AVR4 disulfide bond mutants.

Disrupted Disulfide bond[1]	Cys residue pair[2]	NIA[3]	Stability in AF[4]	Binding to chitin[5]	Stability of chitin-bound AVR4[6]	Identified number of strains[7]
-	-	+++++	+	+	+	ND
[21-27]	2-3	++	+	+	+	0
[57-72]	6-7	-	-	+/-	+/-	0
[11-41]	1-5	-	-	+	+/-	2
[35-80]	4-8	++	-	+	+	6

1) Disruption of one disulfide bond either by a cysteine substitution in PVX::*Avr4* or by partial chemical reduction
2) Successive numbering of the cysteine residues in AVR4 (Fig. 5)
3) Necrosis-inducing activity (NIA) was assayed using PVX-mediated expression of *Avr4* derivatives in MM-Cf4 tomato plants (Fig. 1)
4) AVR4 *des*-species are present (+) or absent (-) after incubation with apoplastic fluid (AF) (Fig. 4)
5) Affinity of AVR4 *des*-species for chitin; +: binds to chitin; +/- :decreased affinity for chitin (Fig. 6)
6) Stability of the AVR4 *des*-species in AF after binding to chitin ; +: stable in the presence of chitin; +/-: partially stable in the presence of chitin (Fig. 7)
7) Number of strains of *Cladosporium fulvum* identified so far carrying a single disulfide bond mutant of AVR4 (Joosten et al., 1994 and 1997).

ND: Not determined

Interestingly, strains of *C. fulvum* producing such a disulfide bond mutant of AVR4 have not been found in nature, possibly due to the fact that modification of [57-72] in AVR4 carries a virulence penalty for *C. fulvum*.

The disulfide bond [21-27] in AVR4 does not seem to contribute to conformational stability of the protein, as AVR4 mutants lacking this bond are not degraded by proteases present in AF (Fig. 4). Moreover, this disulfide bond is absent in proteins of the inv ChBD family, suggesting that it might form a solvent-exposed loop (Fig. 5). Preliminary NMR analysis of AVR4 revealed that the region surrounding Cys[21] adopts an α-helix (C. Spronk and H. van den Burg, unpublished data). As demonstrated previously for a cysteine-knot protein (Darling et al., 2000), the α-helix might increase the stability of this part of AVR4, implying that disulfide bond [21-27] itself is not essential for the conformational stability of AVR4.

When expressed in *Cf-4* tomato plants using the expression vector PVX, the necrosis-inducing activity (NIA) of AVR4 lacking disulfide bond [21-27] was found to be similar to the NIA of AVR4 mutant proteins lacking [35-80]. The NIA of both mutants, however, is less than

that of native AVR4. However, in contrast to disulfide bond [35-80], disulfide bond [21-27] does not contribute to conformational stability of AVR4, suggesting that the mechanisms underlying the reduced NIA of both AVR4 *des*-species are different. Possibly, when AVR4 mutant carrying a disrupted disulfide bond [35-80] is expressed by PVX in tomato, a fraction of the protein is degraded by proteases present in the intercellular space, while the remaining fraction of the protein triggers *Cf-4*-mediated defense responses. The reduced NIA of AVR4 mutant carrying a disrupted disulfide bond [21-27] cannot be explained by sensitivity to proteases, but is most likely due to the amino acid substitution itself or due to a conformational change of the protein. Possibly, the remaining NIA of disulfide bond mutant [21-27] of AVR4 still triggers *Cf-4*-mediated defense responses that restrict growth of *C. fulvum*. This could be a reason why, in spite of the fact that disruption of disulfide bond [21-27] does not affect chitin-binding activity of AVR4, no strains of *C. fulvum* virulent on plant carrying *Cf-4* have thus far been found that carry such a mutation.

In addition to the three Cys substitutions present in the majority of mutants found in nature and discussed above, two other natural amino acid substitutions have been found in AVR4 ($Thr^{37}Ile$ and $Tyr^{38}His$) (Joosten et al., 1997). Both modifications are thought to affect the conformational stability of AVR4, based on a report by Zhu and Braun (1999). The substituted residues are located in strand β2 of the first anti-parallel β-sheet, which is in the core of the protein structure (Suetake et al., 2000). In AVR4, Thr^{37} and Tyr^{38} are putatively paired with Pro^{30} and Tyr^{29} in strand β1, respectively, and these four residues also appear to be conserved in the inv ChBD (Van den Burg et al., 2003). Moreover, Zhu and Braun (1999) showed that in β-strands Pro-Ile cross-strand contact pairs are virtually absent, whereas a Pro-Thr cross-strand contact pair is allowed (Zhu and Braun, 1999). Similarly, aromatic residues are exclusively found at position 38 (Tyr/ Phe/ Trp > 98%) of the inv ChBD. They also showed that hydrophobic residues are the favorite cross-strand contact partners, whereas His residues are not favored as a cross-strand contact partner for these hydrophobic residues. Therefore, we propose that $Thr^{37}Ile$ and $Tyr^{38}His$ substitutions will both lead to a partially destabilization of the anti-parallel β-sheet, which in turn could decrease conformational stability of AVR (See also Chapters 2 and 5).

Altogether, the disulfide bond AVR4 mutants produced by natural strains of *C. fulvum* show increased sensitivity towards proteases present in the apoplast as compared to native AVR4. However, these AVR4 mutant proteins retained the ability to bind to chitin, and moreover, when bound to chitin they are less sensitive to proteolytic breakdown. This suggests that strains virulent on plants carrying *Cf-4* produce disulfide bond mutants of AVR4 that escape degradation by proteases present in the apoplast upon binding to the cell wall of *C. fulvum* at site where chitin is accessible. Thus, while *Cf-4*-mediated resistance is

evaded, the putative intrinsic function of the disulfide bond mutants of AVR4, in protecting the cell wall of *C. fulvum* against plant chitinases, is retained.

Materials and Methods

Construction of PVX derivatives and PVX inoculation procedure

Avr4 mutants encoding various Cys-to-Ala substitutions were generated by PCR-based site-directed mutagenesis on the plasmid pTXΔGC3a, containing the native *Avr4* sequence (Joosten et al., 1997). PCR amplification was carried out using mutagenic primers (Table 4) designed to generate two overlapping PCR fragments that carry the introduced mutation. These PCR fragments were subsequently used in a PCR-mediated overlap extension, using forward primer OX10 and reverse primer N31 (Table 4). The PCR product was digested with *Cla*I, and cloned into the *Cla*I-digested site of the expression vector pTXΔGC3a (Chapman et al., 1992) and sequenced. *In vitro* transcription of the plasmids and subsequent inoculation on *Nicotiana clevelandii* and tomato was performed as described (Joosten et al., 1997). *N. clevelandii* and the tomato (*Lycopersicon esculentum*) cultivars Moneymaker (MM) and the near-isogenic line MM-Cf4 were grown as described (De Wit and Flach, 1979).

Table 4. Mutagenic primers of *Avr4* used to introduce Cys-to-Ala substitutions in PVX::*Avr4*.

Primer	5' 3'
C11Aa	CTT GGG GCT TGG CTG GGT TGT ATG G
C11Ab	CCA TAC AAC CCA GCC AAG CCC CAA G
C21Aa	CCT TGG GAC CCA TGG CCT TGG TGT CG
C21Ab	CGA CAC CAA GGC CAT GGG TCC CAA GG
C27Aa	GGG TTC GGG TAG AGG GCA TCC TTG GGA CCC
C27Ab	GGG TCC CAA GGA TGC CAC TGT CGG GG
C35Aa	GTA TGT AGG TTG TGG CAC TGT CGG GG
C35Ab	CCC CGA CAG TGC CAC AAC CTA CAT AC
C41Aa	CGT CGA GCG GTA CGG CCT GTA TGT AGG
C41Ab	CCT ACA TAC AGG CCG TAC CGC TCG ACG
C57Aa	GCA GTC CTT TTG GGG CTG GCT TAA CCA C
C57Ab	GTG GTT AAG CCA GCC CCA AAA GGA CTG C
C72Aa	GGT TTG GAT AGT CGG CCC ACT TCT TGC C
C72Ab	GGC AAG AAG TGG GCC GAC TAT CCA AAC C
C80Aa	GTC TTT ACC GGG GCC GTA CTC AGG
C80Ab	CCT GAG TAC GGC CCC GGT AAA GAC
OX10	CAA TCA CAG TGT TGG CTT GC
N31	GAC CCT ATG GGC TGT GTT G

Partial reduction and cyanylation of AVR4

Expression of heterologous AVR4 was achieved in the methylotrophic yeast *Pichia pastoris*, and AVR4 was purified from culture fluid (Van den Burg et al., 2001). The disulfide bonds of

AVR4 were partially reduced with TCEP (Sigma) (Wu and Watson, 1997 and 1998). An 0.1 M TCEP stock solution was prepared in 6 M Guanidine-HCl in 0.1 M citrate buffer (pH 3) and stored at –20°C (up to six months without deterioration). For each reduction reaction, 100 μg of native AVR4 was dissolved in 10 μL of 6 M guanidine-HCl in 0.1 M citrate buffer (pH 3). The reaction was initiated by adding a 6-fold molar excess of TCEP to AVR4, following an incubation at 20°C for 15 min. Subsequently, a 80-fold molar excess of CDAP (Sigma) was added to cyanylate the freed thiol groups (15 min, 20 °C, in the dark). The 0.1 M CDAP stock solution in 6M guanidine-HCl in 0.1 M citrate buffer (pH 3) was freshly prepared prior to each reaction.

Reverse-phase high-performance liquid chromatography of the partially reduced AVR4 protein mixture

The TCEP/CDAP reaction mixtures were separated by analytical reverse-phase high performance liquid chromatography (RP-HPLC), using a 150 × 3.9 mm Delta-Pak C18 column (300 Å, 5 μm; Waters). The separation was monitored at 215 nm and predominant peaks were manually collected. HPLC elution solvents consisted of 0.1% (v/v) trifluoracetic acid (TFA) in water (solvent A), and 0.1% (v/v) TFA in acetonitrile (solvent B). HPLC was operated at a flow rate of 1 mL/min. The applied gradient was from 5 to 20% (percentage B in solvent A) in 2 min, from 20 to 30% in 40 min, and from 30% to 60% in 3 min. AVR4 eluted at approximately 25% of solvent B. Integration of the HPLC profile was achieved using the supplied Waters software. Appropriate fractions (containing the AVR4 *des*-species) were lyophilized before storage. All solvents used were HPLC grade.

Base-catalyzed cleavage and identification of the fully reduced peptide fragments by mass spectrometry

Lyophilized HPLC fractions containing the AVR4 *des*-species were dissolved, first, in 2 μL of 1 M NH_4OH, 6 M guanidine-HCl, followed by 5 μL of 1 M NH_4OH, and incubated at 20°C for 1 hr. The excess of NH_4OH was evaporated in a Speed-Vac system for approximately 30 min to obtain an almost dry sample. Subsequently, the remaining disulfide bonds were reduced by adding an excess of TCEP (10 μL of 0.1 M TCEP stock) and the mixture was incubated at 37°C for 30 min. The peptide mixtures were analyzed by MALDI-TOF mass spectrometry (MS) (Perceptive Biosystems Voyager DE-RP). Thereby, small aliquots of the peptide samples were applied to a saturated matrix solution that was freshly prepared (α-cyano-4-hydroxycinnamic acid; Sigma; 10 mg/mL in acetonitrile/water/TFA [50/50/1, v/v/v]) and 1 μL was deposited on a sample plate (Karas and Hillenkamp, 1988; Kussmann et al., 1997). Depicted spectra represent averages of 100 to 256 consecutive laser pulses. The

instrument was generally operated in the positive mode at an acceleration voltage of 23 kV, combined with a delayed extraction. Spectra were externally calibrated with bovine Cytochrome C (12,230.9 Da), bovine Insulin (5,734.6 Da) (both Sigma) and Microperoxidase 8 (1,506.5 Da) (Primus et al., 1999).

Incubation of the AVR4 *des*-species with apoplastic fluid

Apoplastic fluid (AF) was isolated from leaves of MM-Cf4 plants (De Wit and Spikman, 1982) that were infected with strain #38 of *C. fulvum* (race 4) (Joosten et al., 1997). Native AVR4 and AVR4 *des*-species (4 μg) were incubated at 30^{o}C for 1 hr in the presence of 0.1 μL AF (~0.1 μg total protein). Protease inhibitors consist of a standard protein inhibitor cocktail with EDTA (Roche; 1 tablet/ml of AF). Protein samples were separated by tricine SDS-PAGE (Schägger et al., 1987).

The polysaccharide-binding assay

Native AVR4 and AVR4 *des*-species (4 μg) were incubated at room temperature for 1 hr (unless stated otherwise) with an excess of 5 mg of insoluble chitin beads (New England Biolabs) or chitosan (Sigma) in 50 mM Tris·HCl (pH 8) and 150 mM NaCl (500 μL final volume) as described (Van den Burg et al., 2003). The insoluble fraction was precipitated by centrifugation (13,000 x g for 3 min.). The supernatant was recovered and lyophilized. The pellet fraction was boiled in 200 μL 1% SDS to release bound protein and subsequently centrifuged. The retrieved supernatant (containing bound AVR4) fraction as well as the lyophilized supernatant fraction (containing unbound AVR4) was examined for its protein content by tricine SDS-PAGE.

Molecular modeling of AVR4 using the NMR structure of tachycitin

The NMR structure of tachycitin (ID code PDB databank: 1DQC) was used as template structure to model the structure of AVR4 using Modeler 6.1 (Sali and Blundell, 1993; Sali et al., 1995; Fiser et al., 2000). Additional loop refinement was used to model the two relatively large gaps, i.e. 6- and 12 amino acids. The disulfide bonds were fixed during the calculations. A thousand models were constructed, of which the ten lowest scoring structures were further examined. The models were found reliable using standard algorithms (Sippl, 1993; Sanchez and Sali, 1998).

Chapter 5

Structural separation of necrosis-inducing activity and chitin-binding ability within the AVR4 protein

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Structural separation of necrosis-inducing activity and chitin-binding ability within the AVR4 protein

Abstract

The product of avirulence gene *Avr4* of the tomato pathogen *Cladosporium fulvum* has been identified by its ability to elicit *Cf-4*-mediated defense responses, including the hypersensitive response (HR), in tomato. The AVR4 elicitor has also been demonstrated to share structural homology with the chitin-binding domain of chitin-binding proteins of invertebrates (inv ChBD) and to bind to chitin *in vitro*. AVR4 does not require binding to chitin in order to elicit a HR. Here, we analyzed whether domains responsible for necrosis-inducing activity (NIA) and chitin-binding ability within AVR4 can be distinguished. Therefore, we performed a peptide scan (PEPSCAN) analysis using polyclonal antibodies raised against native AVR4 to identify antigenic domains in AVR4 containing putatively solvent-exposed residues that condition NIA. This PEPSCAN identified one major antigenic domain (Cys^{41}-Cys^{57}) and two minor antigenic domains (Ile^{17}-Asn^{31}; Gln^{62}-Asn^{76}) in the AVR4 protein. All single residues, except for Cys, Pro, and Gly, which are present in the major antigenic domain were replaced by Ala, as this domain exhibits the highest affinity for AVR4 antibodies. Moreover, to determine which residues are important for the chitin-binding ability of AVR4, all aromatic residues and two additional residues (conserved in inv ChBDs) were replaced by Ala. We show that substitution of single amino acid residues in the major antigenic domain of AVR4 does not affect its NIA. Substitution of the aromatic residues Tyr^{38} and Trp^{63} by Ala, however, reduced the NIA of AVR4. These AVR4 mutant proteins appear to be unstable, suggesting that Tyr^{38} and Trp^{63} contribute to conformational stability rather than to NIA of AVR4. In this study, no individual amino acid residues could be identified that are essential for binding of AVR4 to chitin, suggesting that multiple rather than single amino acid residues contribute to chitin-binding ability.

Introduction

The product of the avirulence gene *Avr4* of the tomato pathogen *Cladosporium fulvum* has been identified by its ability to confer avirulence to strains of the fungus on tomato plants that carry the matching *Cf-4* resistance gene (Joosten et al., 1994). *Avr4* encodes a pre-pro-protein of 135 amino acids that is targeted to the apoplastic space during growth in tomato leaves and processed by plant and/or fungal proteases into a mature protein of 86 amino

acids (Joosten et al., 1997). No sequence homology was found between AVR4 and other sequences available in databases. A motif search, based on the spacing of the Cys residues and the elucidated disulfide bond pattern of AVR4, revealed that AVR4 shares structural homology with proteins of the invertebrate chitin-binding domain (inv ChBD) family (Van den Burg et al., 2003), including tachycitin of the horseshoe crab *Tachypleus tridentatus* (Kawabata et al., 1996). Although there is no significant amino acid sequence similarity between inv ChBDs and plant ChBDs, a local structural similarity was found between the inv ChBD of tachycitin and the ChBD of the plant chitin-binding protein hevein (Shen and Jacobs-Lorena, 1999; Suetake et al., 2000). For hevein, the aromatic amino acid residues Trp^{21} and Trp^{23} have been identified to establish specific binding to chitin-derived oligosaccharides through hydrophobic interactions (Asensio et al., 1995 and 1998). This binding is further strengthened by hydrogen bonding with residue Ser^{19} of hevein (Asensio et al., 1998). These chitin-binding residues of hevein share a high degree of conservation with hydrophobic and polar residues of ChBDs of invertebrate and plant proteins (Suetake et al., 2000).

Van den Burg et al. (2003) have demonstrated that AVR4 binds to chitin *in vitro* and that it protects both *Trichoderma viride* and *Fusarium solani* f.sp. *phaseoli* against cell wall lysis and growth inhibition by plant chitinases *in vitro*. However, *C. fulvum* is insensitive toward chitinases *in vitro* (Joosten et al., 1995), possibly due to the fact that chitin is not accessible in cell walls of *C. fulvum in vitro* (Van den Burg et al., 2003). As the *Avr4* gene is not expressed during *in vitro*-growth of *C. fulvum* (Joosten et al., 1997), protection against plant chitinases *in vitro* is independent of AVR4. On the other hand, the *Avr4* gene is induced instantly upon ingress of the stomata of tomato leaves and AVR4 was found to accumulate on the cell wall of *C. fulvum in planta,* most likely at sites where chitin is accessible (Joosten et al., 1997; Van den Burg et al., 2003). This implies that during growth of *C. fulvum* in susceptible tomato leaves, AVR4 protects the fungus against plant chitinases. In addition, most AVR4 mutant proteins secreted by strains of *C. fulvum* virulent on plants expressing *Cf-4* are modified in such way that they are degraded by proteases in the apoplastic space of tomato, yet, still exhibit the ability to bind to chitin (Chapter 4). This indicates that AVR4 recognition is evaded without losing the putative intrinsic function of AVR4 in protecting the cell wall of *C. fulvum* against hydrolytic activity of plant chitinases.

In AvrPto, an AVR protein that triggers *Pto*-mediated resistance towards *Pseudomonas syringae* pv. *tomato*, a cluster of mutations has been identified that disrupt its avirulence but not its virulence function (Shan et al., 2000). This structural separation of avirulence and virulence function raised the question whether similar mutations can be identified in AVR4 that abolish its necrosis-inducing activity (NIA) on plants that express *Cf-4*, without affecting

its chitin-binding ability. For AVR9 mutant proteins that exhibit reduced NIA, it has been demonstrated that these mutants also display a reduced affinity for polyclonal antibodies raised against native AVR9, indicating that there is a positive correlation between antigenicity and NIA (Mahé et al., 1998). To determine which amino acid residues in AVR4 are required for its NIA, we performed a peptide scan (PEPSCAN) analysis (Meloen et al., 1995) using polyclonal antibodies raised against native AVR4. This epitope mapping technique identified one major antigenic domain (Cys^{41}-Cys^{57}) and two minor antigenic domains (Ile^{17}-Asn^{31}; Gln^{62}-Asn^{76}). Here, we replaced all single amino acid residues, except for Cys, Pro, and Gly, in the major antigenic domain of AVR4 by Ala, as this domain exhibits the highest affinity for AVR4 antibodies and was therefore thought to contain putatively solvent-exposed amino acid residues that condition NIA of AVR4. Moreover, to determine which residues in AVR4 are important for its chitin-binding ability, all aromatic residues and two additional residues (conserved in inv ChBDs) were replaced by Ala. We show that substitution of single amino acid residues in the major antigenic domain of AVR4 does not affect its NIA. Substitution of the aromatic residues Tyr^{38} and Trp^{63} by Ala, however, reduced the NIA of AVR4. These AVR4 mutant proteins appear to be unstable, suggesting that Tyr^{38} and Trp^{63} contribute to conformational stability rather than to NIA of AVR4. In this study, no AVR4 mutant protein could be identified that exhibited a decreased affinity for chitin, implying that multiple rather than single amino acid residues contribute to chitin-binding ability of AVR4.

Results

The identification of antigenic domains in AVR4

For AVR9 mutant proteins, a positive correlation was found between affinity for polyclonal antibodies raised against native AVR9 and the necrosis-inducing activity (NIA) (Mahé et al., 1998), suggesting that putatively solvent-exposed amino acid residues present at antigenic sites might condition NIA. To identify antigenic domains in AVR4 that might contain residues that are required for its NIA, a peptide scan (PEPSCAN) analysis (Meloen et al., 1995) was performed using polyclonal antibodies raised against native AVR4. Therefore, a series of 77 overlapping peptides of 12 amino acid residues (12-mers), spanning the overall mature AVR4 protein of 86 amino acids, were synthesized on polyethylene pins and used for epitope-mapping by conventional ELISA. This PEPSCAN identified one major antigenic domain (Cys^{41}-Cys^{57}) and two minor antigenic domains (Ile^{17}-Asn^{31} and Gln^{62}-Asn^{76}) in the mature AVR4 protein (Fig. 1).

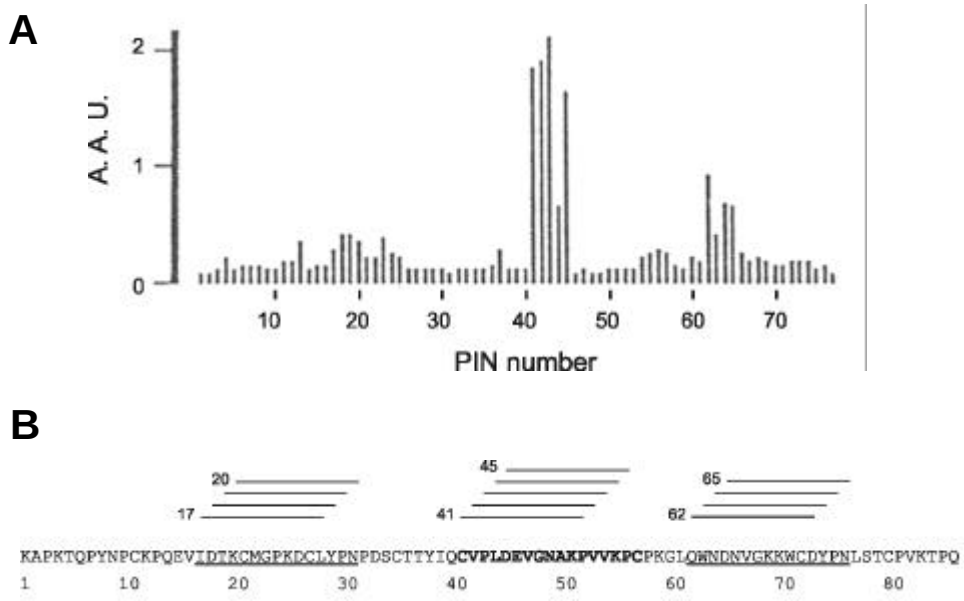

Fig. 1. A peptide scan analysis identified one major and two minor antigenic domains in AVR4. **A,** For epitope-mapping, the mature AVR4 protein of 86 amino acids was subjected to a peptide scan (PEPSCAN) analysis using polyclonal antibodies raised against native AVR4. A series of 77 overlapping 12-mer peptides were synthesized on polyethylene pins and used for epitope-mapping by ELISA) (Meloen et al., 1995). Absorption at 405 nm was determined (A.A.U., arbitrary absorption units). The highest affinity for AVR4 antibody was found for peptides with PIN numbers 41-45, encompassing the so-called major antigenic domain of AVR4 (Cys^{41}-Cys^{57}). AVR4 antibodies also bound to peptides with PIN numbers 17-20 and 62-65, resulting in the identification of two minor antigenic domains (Ile^{17}-Asn^{31} and Glu^{62}-Asn^{76}, respectively). **B,** The major (bold) and the two minor antigenic domains (underlined), as determined by the PEPSCAN, are indicated in the linear amino acid sequence of the mature AVR4 protein. The 12-mer peptides with PIN numbers 17-20, 41-45 and 62-65 are indicated.

To determine whether these linear 12-mers derived from AVR4 can elicit a *Cf-4*-mediated hypersensitive response (HR) upon injection into leaves of plants expressing the corresponding resistance gene *Cf-4*, 12-mers were synthesized but not bound to plastic pins. Moreover, two peptides (15- and 16-mers) spanning the major antigenic domain (Val^{42}-Pro^{56} and Cys^{41}-Pro^{56}) and one 15-mer spanning one of the minor antigenic domains (Gln^{62}-Asn^{76}) were synthesized. None of the linear peptides appeared to induce a HR when injected into leaves of MM-Cf4 plants, not even at 10 μM, which is a 100-fold higher than the concentration used to elicit a HR upon injection of native AVR4 in MM-Cf4 leaves (data not shown). Thus, although antigenic domains might represent domains that are involved in

AVR4 recognition by MM-Cf4 plants, linear synthetic peptides derived from AVR4 do not elicit *Cf-4*-mediated HR.

The necrosis-inducing activity (NIA) of AVR4 mutant proteins

The major antigenic domain of AVR4, as determined by PEPSCAN (Fig. 1), exhibits the highest affinity for AVR4 antibodies and was therefore thought to contain putatively solvent-exposed residues that condition NIA of AVR4. To identify which of these residues are required for NIA of AVR4, amino acid residues present in the major antigenic domain of AVR4 (Asp^{45}, Glu^{46}, Asn^{49}, and Lys^{51}) were replaced, one by one, by Ala. For all other residues present in this domain (Val^{42}, Leu^{44}, Val^{47}, Val^{53}, Val^{54}, and Lys^{55}), it has previously been demonstrated, by PVX-mediated expression of AVR4 mutant protein-encoding genes in plants carrying *Cf-4*, that independent replacement of these residues by alanine did not affect the NIA of AVR4 (Table 1). Moreover, as Cys, Pro and Gly residues are known to affect the overall structure of proteins significantly, these residues were not replaced.

To determine the NIA of the AVR4 mutant proteins, Agrobacterium cultures carrying AVR4 (mutant)-encoding genes were diluted into an Agrobacterium culture carrying the *Cf-4* resistance gene and infiltrated into tobacco leaf sectors. No significant differences in necrosis-inducing activities were observed between the various AVR4 mutant proteins and AVR4 wild-type, demonstrating that residues Asp^{45}, Glu^{46}, Asn^{49}, and Lys^{51} are not essential, at least not individually, for NIA of AVR4 (Table 1).

Interestingly, amongst the AVR4 mutant proteins carrying substitution of amino acid residues that might be required for chitin-binding ability of AVR4 (see below), two mutants, $AVR4^{Y38A}$ and $AVR4^{W63A}$, were identified that showed reduced NIA (Fig. 2). A similar reduced activity was observed for the AVR4 mutant protein carrying a histidine rather than an alanine substitution at position Tyr^{38} (Chapter 2), which is a naturally occurring AVR4 mutant protein secreted by a strain of *C. fulvum* that is virulent on MM-Cf4 plants (Joosten et al., 1997). When apoplastic fluid (AF) isolated from agroinfiltrated tobacco leaves was analyzed by western blotting, it appeared that $AVR4^{Y38A}$ and $AVR4^{W63A}$ are unstable (Fig. 3; Table 1). These data suggest that residues Tyr^{38} and Trp^{63} both contribute to conformational stability rather than to NIA of AVR4.

The chitin-binding ability of AVR4 mutant proteins

To determine whether aromatic residues are required for chitin-binding ability of AVR4, as demonstrated for hevein (Asensio et al. 1995 and 1998), we replaced all individual aromatic residues present in AVR4 (Tyr^{8}, Tyr^{29}, Tyr^{38}, Trp^{63}, Trp^{71} and Tyr^{74}) by Ala. Moreover, we replaced two additional residues in AVR4 (Ser^{34} and Asp^{73}) by Ala, as these are conserved

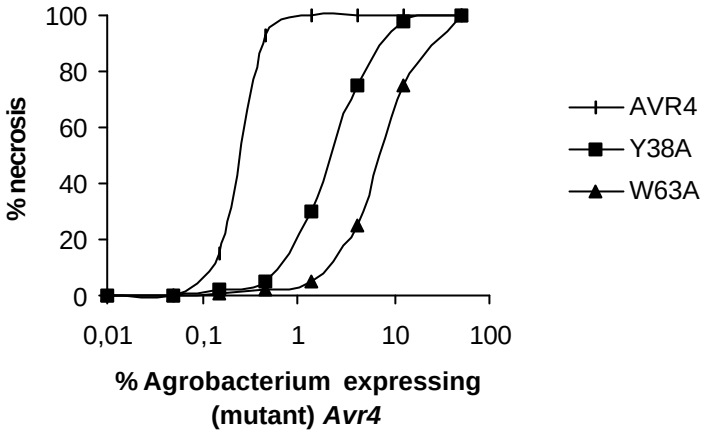

Fig. 2. AVR4^{Y38A} and AVR4^{W63A} mutant proteins exhibit reduced necrosis-inducing activity. The necrosis-inducing activity (NIA) of AVR4^{Y38A} (black square) and AVR4^{W63A} (black triangle) was compared with the NIA of native AVR4 (black diamond). Agrobacterium cultures expressing AVR4 (mutant)-encoding genes were diluted in Agrobacterium cultures expressing the *Cf-4* resistance gene and infiltrated into neighboring tobacco leaf sectors as reported by Van der Hoorn, et al. (2000). The percentage of the infiltrated area that had become necrotic at 7 days post infiltration was measured and plotted against the percentage of Agrobacterium culture expressing the AVR4 (mutant)-encoding gene.

in the chitin-binding domains of chitin-binding proteins of invertebrates (inv ChBDs) (Fig. 4). The AVR4 mutant proteins were transiently expressed by agroinfiltration into tobacco leaves and AF was isolated, three days post infiltration. To determine the chitin-binding ability of these AVR4 mutant proteins, AF was incubated with chitosan (negative control) or chitin for 2 hrs at 22°C. Subsequent centrifugation resulted in a separation between chitin-bound AVR4 (pellet) and free AVR4 (supernatant). Following additional incubation steps (see Materials and Methods), both fractions were subjected to Western blot analysis (Fig. 5). When AVR4 was incubated with chitosan, no AVR4 was detected in the pellet fraction (Fig. 5), indicating that AVR4 does not bind to chitosan, which is consistent with previously obtained results (Van den Burg et al., 2003). Upon incubation with chitin, however, all AVR4 proteins (both native and mutant) appeared to be present in the pellet fraction, indicating that chitin has removed all free AVR4 proteins from the supernatant (Fig. 5). To allow detection of AVR4 mutant proteins that are unstable (AVR4^{Y38A} and AVR4^{W63A}), the exposure of the film to the immunoblot was extended with 15 min. This resulted in detection of bound, but not unbound, AVR4 mutant proteins (Fig. 5). The same result was obtained for

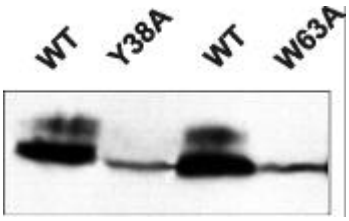

Fig. 3. AVR4^{Y38A} and AVR4^{W63A} mutant proteins are stable. Transient expression of AVR4 (mutant)-encoding genes in tobacco by agroinfiltration was performed to examine the effect of alanine substitutions in AVR4 on protein stability. To allow quantitative comparison of protein levels, neighboring leaf halves were infiltrated with Agrobacterium cultures expressing genes that encode wild-type AVR4 (WT) versus AVR4 mutant (Y38A or W63A) proteins. Three days after infiltration, apoplastic fluid (AF) was isolated and analyzed by western blotting, employing antibodies raised against the double c-Myc tag, which was fused to the N-terminus of the encoded protein. Note that in these AFs the amount of AVR4 mutant proteins is significantly lower than the amount of wild-type AVR4 protein.

AVR4 mutant protein carrying a Tyr^{38}His substitution (data not shown), which is consistent with most other AVR4 mutant proteins that are secreted by strains of *C. fulvum* virulent on MM-Cf4 plants (Chapter 4). Further extension of exposure times did not result in detection of AVR4 mutant proteins in the supernatant fractions. Moreover, AVR4 mutants with mutations in the major antigenic domain also retained the ability to bind to chitin (Table 1). Thus, in this study, no AVR4 mutant proteins with reduced chitin-binding ability could be identified.

Discussion

The *Avr4* gene of *Cladosporium fulvum* encodes an elicitor protein that has been identified by its ability to elicit *Cf-4*-mediated plant defense responses, including a hypersensitive response (HR) (Joosten et al., 1994). Moreover, AVR4 was found to share structural homology with the chitin-binding domain of chitin-binding proteins of invertebrates (inv ChBD) and to bind to chitin *in vitro* (Van den Burg et al., 2003). When transgenic tobacco cell suspensions expressing *Cf-4* are treated with the AVR4 elicitor or when leaves of MM-Cf4 plants are injected with AVR4, active defense responses, including a HR, are induced (Joosten et al., 1994; De Jong et al., 2002a and 2002b). This response is independent of the presence of chitin, demonstrating that AVR4 does not require binding to chitin in order to exhibit necrosis-inducing activity (NIA). Here, we analyzed whether domains responsible for NIA and chitin-binding ability within the AVR4 protein can be distinguished.

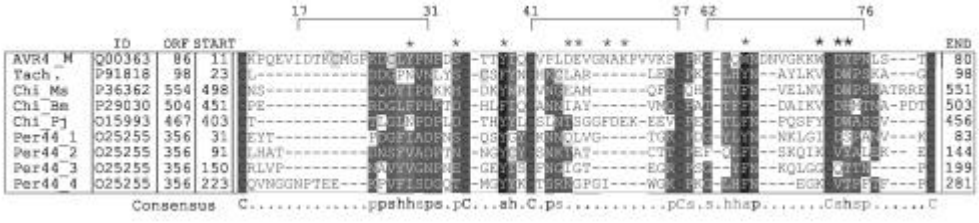

Fig. 4. Sequence alignment between AVR4 and various chitin-binding proteins of invertebrates. The identified disulfide bond pattern and the cysteine spacing of AVR4 was used to perform a motif-based search (URL:**http://motif.genome.ad.jp**) (Van den Burg, et al. 2003). AVR4 was found to contain a chitin-binding domain as found in chitin-binding proteins of invertebrates (inv ChBD). The alignment made in Clustal X shows the following sequences (depicted from top-to-bottom): Mature AVR4 protein of *Cladosporium fulvum*, tachycitin of *Tachypleus tridentatus* (Japanese horseshoe crab), Chitinase of *Manduca sexta* (tobacco hawkmoth), *Brugia malayi* (nematode), *Penaeus japonicus* (prawn), and Peritrophin 44 of *Lucilia cuprina* (fly). The latter sequence contains four inv ChBDs, which represent Per44_1 to Per44_4. The inv ChBD contains six Cys residues that are conserved (black boxed). The consensus is indicated at the bottom (C, cysteine residue; p, polar; s, small residues; h, hydrophobic; a, aromatic). The major antigenic domain (Cys^{41}-Cys^{57}) and the two minor antigenic domains (Ile^{17}-Asn^{31} and Glu^{62}-Asn^{76}), as determined by PEPSCAN (Fig.1), are shown by bars on top of the alignment. Asterisks at the top indicate all residues, except for Tyr^{8}, which have been selected for mutational analysis of native AVR4. ID, ID-code used in the trEMBL database; ORF, total number of residues encoded by the open reading frame (for AVR4 the mature protein is depicted); start/end, represents the first and last amino acid residue of the ChBD, respectively. (This figure has been modified from Van den Burg et al., 2003).

The effect of alanine substitutions in AVR4 on its necrosis-inducing activity (NIA)

Several strains of *C. fulvum* virulent on tomato plants expressing *Cf-4* have been found to secrete AVR4 mutant proteins that carry single amino acid substitutions, including substitutions of cysteine residues that are involved in disulfide bonding in AVR4 (Joosten et al., 1997; Chapter 4). It appeared that these AVR4 mutant proteins are sensitive to proteolytic breakdown in the apoplastic space of tomato, implying that these amino acid substitutions affect the conformational stability of AVR4 (Joosten et al. 1997; Chapter 4). Here, we examined whether AVR4 mutant proteins could be identified that are stable and exhibit reduced NIA. Further, we tested the mutants for their chitin-binding ability (see below). For AVR9 mutant proteins, a positive correlation was found between NIA and antigenicity (Mahé et al., 1998), suggesting that antigenic sites contain putatively solvent-exposed residues that condition its NIA activity. Here, we examined which antigenic domains in AVR4 are recognized by (or bind to) polyclonal antibodies raised against native

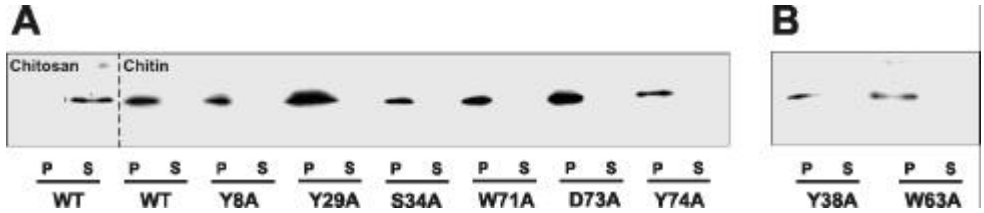

Fig. 5. The introduced alanine substitutions do not affect the chitin-binding ability of AVR4. AVR4 (mutant) proteins carrying alanine substitutions of residues at positions Tyr^{8}, Tyr^{29}, Ser^{34}, Tyr^{38}, Trp^{63}, Trp^{71}, Asp^{73} or Tyr^{74} were tested for their ability to bind to chitin. Apoplastic fluids (AFs), isolated from agroinfiltrated tobacco leaves, containing these AVR4 (mutant) proteins were incubated with 5 mg of chitin at 22°C for 2 hrs. As a negative control, AF containing wild-type AVR4 (WT) was incubated with chitosan instead of chitin. Following centrifugation, free AVR4 (supernatant; s) was separated from chitin-bound AVR4 (pellet; p). Western blot analysis was performed using c-Myc-antibodies. Note that different amounts of AVR4 (mutant) protein are present in these AFs and that, except for the negative control, no AVR4 (mutant) protein is detected in the supernatant fraction. **B,** AFs containing unstable $AVR4^{Y38A}$ and $AVR4^{W63A}$ were analyzed on separate blots, as protein detection required longer exposure of film to the blot.

AVR4. Epitope-mapping employing a peptide scan (PEPSCAN) methodology identified one major antigenic domain (Cys^{41}-Cys^{57}) and two minor antigenic domains (Ile^{17}-Asn^{31} and Gln^{62}-Asn^{76}) in the mature AVR4 protein. It appeared that peptides spanning the individual antigenic domains did not elicit a HR when injected into leaves of MM-Cf4 plants. As demonstrated for most AVR4 mutant proteins produced by *C. fulvum*, cysteine residues are essential for proper conformation and stability of AVR4 (Joosten et al., 1997; Chapter 4). Possibly, linear 12-15-mer peptides derived from AVR4 do not induce a *Cf-4*-mediated HR due to the fact that they lack secondary structures that are required for conformational stability and NIA, thereby rendering the peptides sensitive to proteolytic breakdown in the apoplastic space of tomato leaves.

The major antigenic domain includes a sequence (Val^{42}-Pro^{56}) that is absent in ChBDs of invertebrates (Fig. 4), suggesting that this domain might represents a loop-like structure that is recognized by AVR4 antibodies and possibly also by plants that express *Cf-4.* However, replacement of individual residues in this major antigenic domain of AVR4 by alanine did not affect the NIA of AVR4 mutant proteins. Possibly, a multimeric peptide is responsible for NIA of AVR4, as previously demonstrated for ethylene-inducing xylanase (EIX) elicitor (Rotblat et al., 2002). This implies that multiple, rather than single amino acid replacements are required in the antigenic domain to cause a reduction in NIA of AVR4.

Table 1. The effect of alanine substitutions on necrosis-inducing activity, chitin-binding ability, and stability of AVR4.

	Mutation	NIA	Chitin-binding ability	Stability
AVR4 wild-type	-	+++++	+	+
Major antigenic domain	V42A	+++++	ND	ND
	L44A	+++++	ND	ND
	D45A	+++++	+	+
	E46A	+++++	+	+
	V47A	+++++	ND	ND
	N49A	+++++	+	+
	K51A	+++++	+	+
	V53A	+++++	ND	ND
	V54A	+++++	ND	ND
	K55A	+++++	ND	ND
Aromatic residues	Y8A	+++++	+	+
	Y29A	+++++	+	+
	Y38A	+++	+	-
	W63A	+	+	-
	W71A	+++++	+	+
	Y74A	+++++	+	+
Conserved in ChBD	S34A	+++++	+	+
	D73A	+++++	+	+

Necrosis-inducing activity (NIA) of AVR4 (mutant) proteins was analyzed by transient expression using agroinfiltration into tobacco leaves. Apoplastic fluid isolated from these leaves, containing AVR4 (mutant) proteins, was used in chitin-binding assays and also used to determine protein stability. ND: not determined. ChBD: Chitin-binding domain.

AVR4 mutant proteins carrying a Tyr^{38}Ala or Trp^{63}Ala substitution were found to exhibit reduced NIA. These two AVR4 mutants appear to be unstable, most likely due to proteolytic breakdown, suggesting that, in addition to the Cys residues (Chapter 4), residues Tyr^{38} and Trp^{63} contribute to conformational stability rather than to NIA of AVR4.

The effect of alanine substitutions on chitin-binding ability of AVR4

For hevein, a plant chitin-binding protein, residue Ser^{19} and aromatic residues Trp^{21} and Trp^{23} have been identified to represent an essential part of the ChBD (Cys^{12}-Ser^{32}) (Asensio et al., 1995 and 1998) (Fig. 6). Three-dimensional (3D) structure-based sequence alignment between hevein and inv chitin-binding protein tachycitin revealed that residues Ser^{19}, Trp^{21} and Trp^{23} of hevein correspond to Asn^{47}, Tyr^{49} and Val^{52} of tachycitin, respectively (Suetake et al., 2000). Although AVR4 does not show structural sequence similarity with plant ChBDs, subsequent sequence alignment between tachycitin and AVR4 identified residues Asn^{64}, Gly^{68}, and Trp^{71} to be part of a putative chitin-binding domain of AVR4 (Fig. 6).

```
                                *   *   *
Hevein (12-32)          CPNNLCCS--QW-GWCGST-DEYCS
Tachycitin (40-60)      CPKGLHYN--AYLKVCDW-PSK-AG
AVR4 (57-79)            CPKGLQWNDNVGKKWCDY-PNL-ST
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Fig. 6. Structure-based sequence alignment of tachycitin and AVR4 with the C12-S32 chitin-binding domain of hevein. For hevein, segment C12-S32 was identified as an essential chitin-binding domain. Three-dimensional structure comparison of hevein and tachycitin identified the putative chitin-binding domain of tachycitin. Subsequent amino acid sequence alignment between tachycitin and AVR4 revealed that residues N64, G68, and W71, are part of a putative chitin-binding domain of AVR4. Amino acid residues conserved between the plant, invertebrate and fungal chitin-binding proteins are indicated in bold. Asterisks at the top indicate residues of hevein (S19, W21, and W23) that are essential for its chitin binding.

However, in this study, we have not examined whether residues Asn^{64} and Gly^{68} in AVR4 are essential for its chitin-binding ability. As in AVR4 residue Asn^{64} is in close proximity of residues Asp^{65} and Asn^{66}, which may also contribute to chitin binding through hydrogen bonding, multiple rather than single amino acid substitutions might be required to abolish chitin-binding ability of AVR4. The involvement of residue Gly^{68} was not examined as this residue is thought to be required for structural integrity of AVR4 and, moreover, is not conserved in inv ChBDs.

Here, we examined whether the aromatic residues and two additional residues (Ser^{34} and Asp^{73}), which are conserved in inv ChBDs, are involved in binding of AVR4 to chitin. Although most selected residues (Tyr^{29}, Ser^{34}, Tyr^{38}, Trp^{63}, Asp^{73}, and Tyr^{74}) are conserved in inv ChBDs, all of the AVR4 mutant proteins retained their ability to bind to chitin. Moreover, AVR4 mutant proteins with an alanine substitution at position Asp^{45}, Glu^{46}, Asn^{49}, or Lys^{51} in the major antigenic domain of AVR4, also retained chitin-binding ability. In addition, a histidine substitution of residue Tyr^{38}, as found in an AVR4 mutant protein secreted by a strain of *C. fulvum* virulent on MM-Cf4 plants, did not affect the chitin-binding ability of AVR4. Thus, similar to strains of *C. fulvum* that produce AVR4 mutant proteins carrying a disrupted disulfide bridge (Chapter 4), this strain also circumvents *Cf-4*-mediated resistance by secretion of an unstable AVR4 mutant protein that is still capable of binding to chitin.

Strikingly, although we performed a selective alanine substitution analysis, no AVR4 mutant proteins were identified with decreased affinity for chitin. Possibly, residues other than those selected by us are required for binding of AVR4 to chitin *in vitro*. On the other

hand, to preserve its chitin-binding ability, AVR4 might respond to evolutionary changes in the cell wall composition of *C. fulvum* by adaptive substitution of amino acid residues in the putative ChBD of AVR4, suggesting that AVR4 has the ability to encounter functional modifications. Nevertheless, disruption of disulfide bond Cys^{57}-Cys^{72} in AVR4 reduced its affinity for chitin, as a significant amount of this AVR4 mutant protein was found to be free of chitin in the supernatant fraction (Chapter 4). The major difference between the two reported assays concerns the ratio between AVR4 and chitin used in the chitin-binding assays. Here, we used 75-100 ng of AVR4 in our chitin-binding assay, whereas in Chapter 4 a 40-fold higher concentration of AVR4 (4μg) was used. Moreover, when prolonged incubation times were used, AVR4 mutant protein could no longer be detected in the supernatant fraction. Thus, in our study, shorter incubation times together with an excess of AVR4 mutant protein in relation to chitin might identify amino acid residues that are essential for chitin-binding ability of AVR4.

Although single amino acid mutations were identified that abolished the conformational stability of AVR4, no modifications were found that resulted in a stable AVR4 mutant proteins that exhibit reduced NIA. Possibly, to affect the AVR4 biologically active site that is recognized by *Cf-4* plants, multiple rather than single amino acids need to be substituted, in particular the residues present in the major and minor antigenic domains. As multiple residues are conserved in the chitin-binding domain, more than one residue is thought to condition the ability of AVR4 to bind to chitin. It could be possible that binding of AVR4 to chitin inhibits the NIA of AVR4, which would imply that residues required for chitin binding of AVR4 are also required for its NIA. On the other hand, when bound to chitin, the biologically active site of AVR4 might not be accessible to elicit a *Cf-4*-mediated HR. Altogether, subsequent multiple amino acid substitutions in AVR4 might elucidate whether domains responsible for NIA and chitin-binding ability within the AVR4 protein can be distinguished.

Materials and Methods

Plant materials

Tomato (*Lycopersicon esculentum*) cultivar Moneymaker (MM), near-isogenic line MM-Cf4 (containing an introgession segment that carries the *Cf-4* locus) and tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) plants were grown under standard greenhouse conditions.

PEPSCAN of the mature AVR4 protein

To identify linear epitopes derived from AVR4 recognized by polyclonal antibodies raised against native AVR4, the mature AVR4 protein of 86 amino acids was subjected to a peptide

scan (PEPSCAN) analysis. A set of 77 peptides corresponding to 12-mers spanning the mature AVR4 protein, each peptide overlapping the previous peptide with 11 amino acid residues, was synthesized on solid (polyethylene) support pins (at the COOH-terminus of each amino acid sequence) (Meloen et al., 1995; Williams et al., 1998). The pins with bound peptide were placed into individual wells of a 96-well microtitre plate and an ELISA procedure was carried out, according to Geysen et al. (1987). Briefly, nonspecific binding sites on the pins were blocked by incubation in blocking buffer (PBS containing 5% ovalbumin, 5% horse serum and 1% (v/v) Tween-80) for 30 min at room temperature. Polyclonal antibodies raised against native AVR4 (mouse) was diluted in blocking buffer (1:1000) and incubated overnight at 4°C. After washing with PBS containing 1% (v/v) Tween-80, the pins were transferred to wells containing goat-anti-mouse immunoglobulin horse radish peroxidase conjugate. After incubation for 1 hr at 37°C, unbound antibody was removed from the supports and antibodies were detected by incubation in fresh substrate solution (50 mg ABTS and 20 µl of 30% (w/w) H_2O_2 solution in 100 ml of 0.1 M sodium phosphate/0.08 M citrate buffer, pH 4). Plates were read at 405 nM.

Construction of plasmids for agroinfiltration

To facilitate transient expression of AVR4 mutant proteins by agroinfiltration, binary plasmids were constructed. For site-directed mutagenesis, two PCR fragments were obtained, making use of primers to introduce mutations and specific restriction sites (Table 2). These PCR fragments were subsequently used in overlap extension PCR to amplify a PCR product that contained the open reading frame (ORF) of the *Avr4* (mutant) gene. Two methods were carried out to introduce an alanine substitution at position Ser^{78}, which abolished the putative *N*-glycosylation site of AVR4. Following method (1), overlap extension PCR was performed with forward primer XF7 (introduction of a *Xho*I site) and reverse primer B20-C2 (introduction of a *Pst*I site and the Ser^{78}-to-Ala mutation) (Table 2), using PVX::*Avr4* as a template (Joosten et al., 1997). Following method (2), overlap extension PCR was performed with forward primer A4-XN (introduction of a *Xho*I site) and reverse primer A4-BC (introduction of a *BamH*I site), using pPIC-A4 (Chapter 3) as a template. The PCR products were subsequently digested with *Xho*I/ *Pst*I (1) or with *Xho*I/ *BamH*I (2), cloned into either *Xho*I/ *Pst*I-digested pRH80 (Van der Hoorn et al., 2000) or *Xho*I/ *BamH*I-digested pBluescript SK^+ (Amersham-Pharmacia, Buckinghamshire, UK), respectively, and sequenced. Following subsequent digestion, the ORFs were cloned into *Xho*I/ *EcoR*I-digested pRH298 (Van der Hoorn, unpublished data) or *Xho*I/ *BamH*I-digested pNW30, respectively (Chapter 2). The binary plasmids pRH298 and pNW30 both carry the Cauliflower Mosaic Virus (CaMV) 35S-promoter and the Pi-II terminator (An et al., 1989).

The ORFs of (mutant) *Avr4* were cloned downstream of a double c-Myc-tag, which itself is located downstream of the PR-1a signal peptide for extracellular targeting (Hammond-Kosack et al., 1995).

Table 2. Primers used to introduce mutations and to generate PCR fragments encoding AVR4 (mutant) proteins. Forward (f) and reverse (r) primers and mutations (bold) are indicated and restriction sites are underlined.

Primer		Sequence (5' to 3')
XF7 (*Xho*I site)	(f)	TAGCTCGAGCAAGGCCCCCAAAACTCAACC
B20-C2 (*Pst*I site)	(r)	AAAACTGCAGTCATTGCGGCGTCTTTACCGGACACGTGGCCAGGTTTGGATA
A4-XN (*Xho*I site)	(f)	TAGCTCGAGCAAGGCCCCCAAAACTCAACC
A4-BC (*BamH*I site)	(r)	CGCGGATCCCTATTGCGGCGTCTTTACCG
Y8A	(f)	CTCAACCA**GCC**AACCCATG
	(r)	CATGGGTT**GGC**TGGTTGAG
Y29	(f)	GATTGTCTC**GCC**CCGAACCCCG
	(r)	CGGGGTTCGG**GGC**GAGACAATC
S34A	(f)	GAACCCCGAC**GCC**TGTACAACC
	(r)	GGTTGTACA**GGC**GTCGGGGTTC
Y38A	(f)	GTACAACC**GCC**ATACAGTGTG
	(r)	CACACTGTAT**GGC**GGTTGTAC
D45A	(f)	CCGCTC**GCC**GAAGTTGGCAAT
	(r)	ATTGCCAACTTC**GGC**GAGCGG
E46A	(f)	CCGCTCGAC**GCC**GTTGGCAAT
	(r)	ATTGCCAAC**GGC**GTCGAGCGG
N49A	(f)	GTTGGC**GCC**GCGAAGCCTGTG
	(r)	CACAGGCTTCGC**GGC**GCCAAC
K51A	(f)	GTTGGCAATGCG**GCC**CCTGTG
	(r)	CACAGG**GGC**CGCATTGCCAAC
W63A	(f)	GGACTGCAG**GCC**AACGATAACG
	(r)	CGTTATCGTT**GGC**CTGCAGTCC
D73A	(f)	GAAGTGGTGC**GCC**TATCCAAACC
	(r)	GGTTTGGATA**GGC**GCACCACTTC
Y74A	(f)	GTGGTGCGAC**GCC**CCAAACCTG
	(r)	CAGGTTTGG**GGC**GTCGCACCAC

Agroinfiltration and analysis of apoplastic fluids

Agroinfiltration of transgenic cultures of *Agrobacterium tumefaciens* carrying various AVR4 (mutant)-encoding constructs into *Nicotiana tabacum* cv. Petite Havana SR1 was performed as described in Chapter 2. Apoplastic fluids (AFs) were isolated from agroinfiltrated tobacco leaves, 3 days post infiltration (dpi) and freeze-dried (Chapter 2). Western blot analysis of AFs containing the AVR4 mutant proteins was performed using c-Myc-antibodies (Chapter 2), as mutations in the major antigenic domain of AVR4 might interfere with the affinity for polyclonal antibodies raised against native AVR4.

To test the necrosis-inducing activity (NIA) of AVR4 (mutant) proteins, Agrobacterium cultures carrying AVR4 (mutant)-encoding genes were diluted in an Agrobacterium culture carrying the *Cf-4* resistance gene and infiltrated into leaf sectors of tobacco.

Chitin-binding assay

Aliquots of AFs (100 µl), containing approximately 75-100 ng of AVR4 (mutant) protein, were freeze-dried and resuspended in 500 µl of chitin-binding buffer (ChBB) (50mM Tris/Hcl pH8.0 and 100 mM NaCl). Samples were supplied with approximately 5 mg of chitin (purified chitin powder from crab shells [Sigma-Aldrich, Zwijndrecht, The Netherlands]) and incubated at 22°C for 2 hrs under continuous rotation. As a negative control, AFs were incubated with chitosan (from crab shells [Sigma-Aldrich, Zwijndrecht, The Netherlands]) (Van den Burg et al., 2003). After incubation, samples were centrifuged for 10 min at 13,000 x rpm in a microfuge to separate chitin-bound AVR4 (pellet) from free AVR4 (supernatant). The supernatant was subsequently freeze-dried and the pellet fraction was washed 3 times with ChBB (500 µl) to remove all free AVR4 (mutant) protein. To dissociate bound AVR4 (mutant) protein from chitin or chitosan, the pellet fractions were resuspended into 150 µl of 1% (w/v) SDS and incubated at 95°C for 5 min. Following incubation, samples were centrifuged and supernatant, containing dissociated "bound" AVR4 (mutant) protein, was freeze-dried. Freeze-dried fractions containing unbound AVR4 and bound AVR4 (mutant) proteins were resuspended in 1x SDS-loading buffer (10 mM Tris/HCl pH6.8, 1% (w/v) SDS, 2% (v/v) glycerol, 1% 2-mercaptoethanol and 0.0005 % bromophenol blue) (50 µl) and 10 µl was run on SDS-PAGE. Proteins were blotted on nitrocellulose membranes and the c-Myc-tagged AVR4 (mutant) proteins were detected using Super Signal Chemiluminescent Substrate (pico ECL kit) (Pierce, Rockford, USA), as described in Chapter 2. Kodak X-OMAT films were exposed to the blots for 2-5 min or, occasionally, for 20 min.

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Chapter 6

Summarizing discussion

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The majority of proteins produced by pathogens during growth on susceptible plants are thought to play a role in pathogenesis. Some of these proteins, the products of avirulence (*Avr)* genes, have been demonstrated to trigger defense responses in resistant plants that carry the matching resistance (*R*) genes, thereby causing avirulence of the pathogenic isolates producing these AVR proteins. As a result of selection pressure, pathogens have developed a variety of different strategies to modify these AVRs and thereby evade the induction of plant defense responses. Plants, however, have developed various surveillance and defense mechanisms to withstand the persistent attacks by continuously changing pathogen populations. Most *R* genes are members of complex loci and recombination between alleles and sometimes between different homologues are thought to mediate generation of new resistance specificities (Hammond-Kosack and Jones, 1997; Parniske et al., 1997; Richter and Ronald, 2000). Once activated by AVRs directly or indirectly, R proteins are extremely efficient in inducing plant defense responses that restrict further growth of invading pathogens (Hammond-Kosack and Jones, 1996).

Resistance specificities conferred by homologues of *Cladosporium fulvum* resistance gene *Cf-9*

Within the *Hcr9* (homologues of *Cladosporium R* gene *Cf-9*) gene family of tomato, three homologues have thus far been identified that confer resistance towards *C. fulvum.* The homologues *Hcr9-4D, Hcr9-4E* and *Hcr9-9C*, which represent the *Cf-4, Cf-4E* and *Cf-9* genes, respectively, mediate the induction of defense responses upon recognition of race-specific elicitors of *C. fulvum*, AVR4, AVRE, and AVR9, respectively (Thomas et al., 1998; Joosten and De Wit, 1999) (Chapters 1 and 2). Although the various *Hcr9* homologues share a high degree of sequence homology, all other *Hcr9* homologues identified so far, are non-functional in conferring full resistance towards *C. fulvum* (Parniske et al., 1997; Thomas et al., 1997). The sequence variation in the *Hcr9*s, which mainly occurs in the N-terminal domain and particularly in the solvent-exposed residues of the â-sheets in the leucine-rich repeat (LRR) domains of the encoded proteins, is thought to determine the distinct resistance specificities of the functional Hcr9s. Indeed, gene shuffling and domain swap experiments between *Cf-4* and *Cf-9* revealed that these variant residues are important for Cf-4 and Cf-9 function (Van der Hoorn et al., 2001; Wulff et al., 2001). Van der Hoorn et al. (2001) also demonstrated that it is possible to construct a chimeric Cf-4 protein that carries most of the specificity determinants of Cf-9, with the exception of three solvent-exposed amino acid residues. However, the number of LRRs, which distinguishes Cf-4 and Cf-9 (25

and 27 LRRs, respectively), is essential for Cf-4 and Cf-9 function and can therefore not be changed (Van der Hoorn et al., 2001). As Hcr9-4E and Cf-9 carry the same number of LRRs, this implies that by gene shuffling and domain swaps, a chimeric Cf protein might be generated that exhibits dual recognition specificity towards both AVR4E and AVR9.

A striking feature observed for AVR-induced defense responses is that plants carrying the functional *Hcr9* gene respond in a highly sensitive manner to low amounts of the corresponding AVR protein (Van den Ackerveken et al., 1992; Takken et al., 1999). This implicates that the induction of plant defense responses is mediated by receptors that exhibit high affinity for these AVR elicitor proteins (see below). Previously, early defense responses have been studied in transgenic tobacco cell suspensions that express *Cf-4* or *Cf-9* (Piedras et al., 1998; De Jong et al., 2002b). These studies revealed that both in *Cf-4*- and *Cf-9*-cell suspensions medium alkalization and an oxidative burst are induced upon treatment with AVR4 and AVR9, respectively. The induction of these responses is suppressed at 33°C (De Jong et al., 2002b). Moreover, when *Cf-4*- and *Cf-9*-mediated defense responses are compared, no significant differences in expression of genes that are involved in downstream signaling could be detected (Cai et al., 2001). Nevertheless, distinct necrotic patterns developed in tomato seedlings that express both *Cf-4* and *Avr4* as compared to seedlings that express both *Cf-9* and *Avr9* (Cai et al., 2001). As AVR4E was found to exhibit necrosis-inducing activity (NIA) in tobacco in the presence of Hcr9-4E (Chapter 2), it would be interesting to employ tobacco cell suspensions that carry *Hcr9-4E*, in addition to *Cf-4* and *Cf-9*, to identify differences and similarities in activation of *Hcr9*-mediated signaling pathways. The recent introduction of novel approaches including transcriptomics and proteomics combined with transient silencing assays and mass-spectrometry (Romeis, 2001a) will be instrumental to elucidate novel components in downstream signaling pathways that lead to resistance.

Phylogenic relationships between isolates of *C. fulvum*

During growth in the apoplastic space of tomato, *C. fulvum* secretes a variety of different race-specific AVR proteins and non-race-specific extracellular proteins (ECPs), all of which are small, cysteine-rich, and stable proteins that do not share sequence homology (Joosten and De Wit, 1999). The AVRs and ECPs have been shown to act as elicitor proteins in tomato plants that carry the matching *R* gene (Joosten and De Wit, 1999). Natural strains of *C. fulvum* were found to evade triggering of plant defense responses by modifying *Avr* gene products. Unlike the modifications found in *Avr* genes, thus far only silent mutations have been identified in the open reading frame (ORF) of the virulence gene *Ecp2* in natural strains of *C. fulvum* (M.J.D. de Kock, personal communication). On plants carrying *Cf-ECP2*,

the virulence factor ECP2 behaves as an avirulence determinant (Laugé et al., 1998b). Possibly, there might have been a lack of selection pressure on *C. fulvum* to overcome ECP2 recognition, as, opposed to the *Cf* resistance traits, *Cf-ECP2* has not yet been introduced in commercially grown tomato cultivars (Chapter 1).

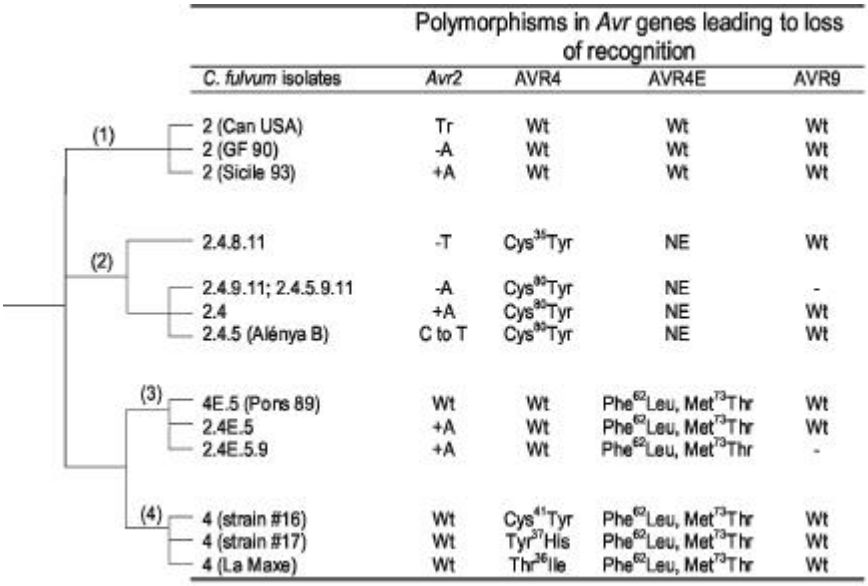
Within the *C. fulvum* population, several isolates have been identified that evade more than one *Cf* resistance trait. Subsequent sequence analysis of the corresponding *Avr* ORFs revealed that *C. fulvum* employs a variety of different mechanisms to evade *Cf*-mediated resistance. Evasion of *Cf-2*-mediated resistance by *C. fulvum* is enabled by a retrotransposon insertion, or by several different single base pair deletions or insertions in the *Avr2* ORF, resulting in the production of truncated AVR2 proteins (Luderer et al., 2002b). Strains of *C. fulvum* evade *Cf-4*-mediated resistance by production of a truncated AVR4 protein, or by secretion of unstable AVR4 mutant proteins that carry different single amino acid substitutions. In the ORF of the *Avr4E* gene present in strains virulent on plants carrying *Hcr9-4E*, a double point mutation was found, resulting in a two amino acid substitutions, $Phe^{62}Leu$ and $Met^{73}Thr$ ($avr4E^{LT}$). This elicitor-inactive mutant protein is stable and the single amino acid substitution $Phe^{62}Leu$ in AVR4E appears to be sufficient to evade *Hcr9-4E*- mediated resistance (Chapter 2). Moreover, some strains virulent on *Hcr9-4E* plants have been identified that carry a wild-type *Avr4E* ORF, yet, produce no functional AVR4E elicitor protein due to lack of *Avr4E* gene expression (Chapter 2). Finally, all strains of *C. fulvum* that are virulent on tomato plants carrying the *Cf-9* gene lack the entire *Avr9* ORF (Van den Ackerveken et al., 1992).

AFLP analysis on DNA of *C. fulvum* revealed that lack of sufficient polymorphism amongst isolates within the world-wide *C. fulvum* population did not allow construction of a reliable phylogenetic tree (Joosten and De Wit, 1999). In spite of this limitation, mutations identified in the ORF of *Avr2*, *Avr4*, *Avr4E* and *Avr9* were used to obtain a simplified phylogenetic relationship between *C. fulvum* isolates carrying these mutations (See also Chapter 1; Table 2). As we excluded isolates for which not all four *Avr* ORFs were sequenced, this analysis represents only a small fraction (fourteen) of the isolates present in our worldwide *C. fulvum* collection. Clustering resulted in a so-called dendrogram, which grouped fourteen strains into four main clusters (phenons) (Fig. 1). Phenon 1 includes strains that are virulent on *Cf-2* plants (i.e. races 2) and avirulent on plants that carry *Cf-4*, *Hcr9-4E* or *Cf-9*. This suggests that these strains have thus far only endured selection pressure on AVR2 to overcome *Cf-2*-mediated resistance. Phenon 2 represents strains that have endured selection pressure on AVR2, AVR4 and AVR4E to overcome *Cf-2*, *Cf-4* and *Hcr9-4E*-mediated resistance, respectively (i.e. races 2.4) (Fig. 1). Strains virulent on plants carrying the complete *Cf-4* locus are referred to as races 4, while strains virulent on plants

carrying *Hcr9-4D* or *Hcr9-4E* are referred to as races 4D and races 4E, respectively. A total of 17 breeding lines that carry *Cf-4* were also found to carry *Hcr9-4E* (Haanstra et al., 2000), suggesting that the selection pressure to overcome *Cf-4* is comparable to the selection pressure to overcome *Hcr9-4E*. Thus, while the presence of *Cf-4* is associated with *Hcr9-4E*, plants might exist that carry *Hcr9-4E* but not *Cf-4.* This would explain why, opposed to races 4 and races 4E, thus far no races 4D have been identified. In phenon 2, all strains of *C. fulvum* are clustered that lack *Avr4E* gene expression (NE), indicating that these strains might have a common ancestor carrying a mutation in the promoter sequence of *Avr4E* (Chapter 2). Possibly, following evasion of *Hcr9-4E*-mediated AVR4E recognition, two strains emerged, one with a Cys^{35}-to-Tyr and the other with a Cys^{80}-to-Tyr substitution. These two strains might have endured consecutive selection pressure to overcome *Cf-2*-mediated AVR2 recognition (race 2.4, race 2.4.8.11 and race 2.4.5), as well as *Cf-9*-mediated AVR9 recognition (race 2.4.5.9.11 and 2.4.9.11). There is prove that *Cf-8* is allelic to *Cf-4* and that plants carrying *Cf-11* also carry *Cf-4* (Haanstra et al., 2000), suggesting that races 8 are identical to races 4 and that races 11 require additional modification of the *Avr4* gene product to enable growth on plants carrying *Cf-11.* The strains clustered in phenon 3 all carry the same modification in the *Avr4E* gene product (resulting in $avr4E^{LT}$) and are virulent on plants carrying *Cf-5* (Fig. 1). Race 2.5.9 might have evolved from race 2.5, which in turn might have evolved from race 5. To be able to grow on plants that carry the complete *Cf-4* locus, strains of *C. fulvum* require modifications of both the *Avr4* and *Avr4E* gene products. Phenon 4 includes strains in which both these gene products are modified and as a result, these strains are virulent on plants that carry both *Cf-4* and *Hcr9-4E*. The low genetic variability observed in *Avr4E* suggests that these strains might have diverged from a common ancestor carrying $avr4E^{LT}$. However, as single amino acid substitution $Phe^{62}Leu$ is sufficient to lose elicitor activity of AVR4E (Chapter 2), strains carrying $avr4E^{LT}$ might have evolved from strains carrying the single amino acid substitution $Met^{73}Thr$, but within the *C. fulvum* population, no such strains have thus far been identified.

The intrinsic function of AVR4

The AVR4 protein was found to share structural homology with the chitin-binding domain of chitin-binding proteins of invertebrates (inv ChBD) (Van den Burg et al., 2003). AVR4 has been demonstrated to bind to chitin *in vitro,* but not to other polysaccharides . Moreover, an AVR4-specific high-affinity binding site (HABS) was found in fungal extracts derived from *C. fulvum* mycelium grown *in vitro* (Chapter 3). This AVR4-HABS appeared to be heat and proteinase K resistant and was therefore thought to represent a nonproteinaceous, possibly chitin-like, component. However, dissociation of AVR4 from its HABS was accomplished



	C. fulvum isolates	*Avr2*	AVR4	AVR4E	AVR9
		Polymorphisms in *Avr* genes leading to loss of recognition			
(1)	2 (Can USA)	Tr	Wt	Wt	Wt
	2 (GF 90)	-A	Wt	Wt	Wt
	2 (Sicile 93)	+A	Wt	Wt	Wt
(2)	2.4.8.11	-T	$Cys^{35}Tyr$	NE	Wt
	2.4.9.11; 2.4.5.9.11	-A	$Cys^{80}Tyr$	NE	-
	2.4	+A	$Cys^{80}Tyr$	NE	Wt
	2.4.5 (Alénya B)	C to T	$Cys^{80}Tyr$	NE	Wt
(3)	4E.5 (Pons 89)	Wt	Wt	$Phe^{62}Leu$, $Met^{73}Thr$	Wt
	2.4E.5	+A	Wt	$Phe^{62}Leu$, $Met^{73}Thr$	Wt
	2.4E.5.9	+A	Wt	$Phe^{62}Leu$, $Met^{73}Thr$	-
(4)	4 (strain #16)	Wt	$Cys^{41}Tyr$	$Phe^{62}Leu$, $Met^{73}Thr$	Wt
	4 (strain #17)	Wt	$Tyr^{37}His$	$Phe^{62}Leu$, $Met^{73}Thr$	Wt
	4 (La Maxe)	Wt	$Thr^{36}Ile$	$Phe^{62}Leu$, $Met^{73}Thr$	Wt

Fig. 1. Phylogenetic relationships between *C. fulvum* isolates, based on variation observed in sequences of wild-type and mutant *Avr* ORFs. The open reading frames (ORFs) of *Avr2, Avr4, Avr4E* and *Avr9* genes present in several natural strains of *C. fulvum* avirulent or virulent on plants carrying *Cf-2, Hcr9-4D, Hcr9-4E* or *Cf-9* (wild-type versus races 2, 4D, 4E or 9, respectively) have been sequenced (See also Chapter 1). Strains indicated by Gattieres Furon 90 (GF 90), Alénya B, Pons 89 and La Maxe originate from France. For most other strains the origin is unknown. Mutations in the *Avr2* ORF involve a transposon insertion (Tr), single base pair deletions (-) or insertions (+) in the codon of Lys^{23} or a nucleotide exchange (C to T). The mature AVR4 mutant proteins carry a substitution Tyr-to-Cys at position 35, 41 or 80 (TGT to TAT), or a substitution Tyr^{37}-to-His (TAC to CAC) or Thr^{36}-to-Ile (ACC to ATC). Strains have been identified that lack *Avr4E* gene expression (NE). A double point mutation in the *Avr4E* ORF results in amino acid substitution Phe^{62}-to-Leu (TTT to CTT) and Met^{73}-to-Thr (ATG to ACG) in mature AVR4E. Strains virulent on plants carrying *Cf-9* do not produce the AVR9 protein (-) due to the absence of the entire *Avr9* ORF. Wt: wild-type.

under the same conditions as association, whereas elution of AVR4 from chitin required extreme conditions, such as boiling in 1% SDS. Moreover, affinity crosslinking experiments revealed that AVR4 binds to a fungal component of about 75 kDa, suggesting that the AVR4-HABS represents a proteinaceous compound rather than a polysaccharide such as chitin. Previously, receptor-like proteins of similar sizes (MW between 75 and 85 kDa) have

been identified in plasma membranes of plant cells that exhibit chitin-binding properties (Shibuya et al., 1993; Baureithel et al., 1994; Ito et al., 1997; Kim et al., 2000; Day et al., 2001). As chitin- and glycan polysaccharides are the major components present in fungal cell walls, it could be possible that fungi also carry proteins that exhibit chitin-binding properties. In that case, AVR4 might bind to chitin and this complex might in turn be crosslinked to a fungal chitin-binding protein of about 75 kDa.

AVR4 protects the cell wall of *Trichoderma viride* and *Fusarium solani* f. sp. *phaseoli* against lysis by plant chitinases *in vitro* (Van den Burg et al., 2003). However, *in vitro*-grown *C. fulvum* is insensitive towards the hydrolytic action of plant endoglucanases and chitinases (Joosten et al., 1995), possibly due to the fact that chitin is not accessible in the cell walls of the fungus grown *in vitro* (Van den Burg et al., 2003). However, during growth of *C. fulvum* in the apoplastic space of tomato, AVR4 was found to accumulate on the cell wall of the fungus, most likely at sites where chitin is exposed and accessible (Van den Burg et al., 2003). This is consistent with the observation that no *Avr4* gene expression could be detected during growth of *C. fulvum in vitro*, whereas *Avr4* expression is induced instantly after penetration of tomato leaves (Joosten et al., 1997). Thus, the situation *in vitro* seems quite different from that *in planta,* suggesting that binding of AVR4 to the cell wall of *C. fulvum* protects the fungus against plant chitinases during colonization of tomato (Van den Burg et al., 2003).

A similar counter-defensive strategy has also been reported for the oomycete *Phytophthora sojae*, which secretes glucanase inhibitor proteins (GIPs) that also accumulate on the surface of walls of *Phytophthora* species (Ham et al., 1997; Shiraishi et al., 1997; Rose et al., 2002). These GIPs were found to inhibit the endoglucanases activity of soybean, thereby providing protection against mycelial cell lysis by soybean endoglucanases. Moreover, GIPs suppress the release of glucan oligosaccharide elicitors from oomycetous cell walls and, as a consequence, the activation of plant defense responses normally induced by these oligosaccharides (Côté and Hahn, 1994; Ebel and Cosio, 1994). In the case of AVR4, interaction with chitin could potentially reduce the release of chitin oligosaccharide elicitors, thereby suppressing induction of plant defense responses mediated by putative plasma membrane-localized high-affinity binding sites (HABS) (Baureithel et al., 1994; Ito et al., 1997; Day et al., 2001). On the other hand, unbound AVR4 could potentially inhibit the activation of plant defense responses by interacting with these released chito-oligomers. However, as these plasma membrane-associated HABSs exhibit significantly higher affinities for chito-oligomers as AVR4, AVR4 is not expected to play an active role in suppressing plant defense responses but is rather

expected to have a passive role in protecting *C. fulvum* against cell wall degradation by plant chitinases (Van den Burg et al., 2003).

Perception of AVR4 by resistant plants carrying *Cf-4*

When transgenic tobacco cell suspensions expressing *Cf-4* are treated with AVR4 or when leaves of tomato plants carrying *Cf-4* are injected with AVR4, active defense responses are induced, including a hypersensitive response (HR) (Joosten et al., 1994; De Jong et al., 2002b). This response appeared to be independent of the presence of chitin, demonstrating that AVR4 does not require binding to chitin in order to exhibit (NIA).

Various AVR proteins have been demonstrated to suppress (basal) plant defense responses by interacting with host-encoded proteins, the so-called putative virulence targets (Chapter 1). In resistant plants, however, this interaction is thought to be sensed by R proteins, which subsequently induce downstream defense pathways (Chapter 1). To examine the mechanism(s) of AVR4 perception by resistant plants, binding studies were performed on microsomal membrane fractions (MFs) of plants carrying *Cf-4* that were grown under contained conditions, in order to prevent contamination by saprophytes or pathogens (Chapter 3). It appeared that optimized binding conditions that allowed detection of an AVR9-HABS (Kooman-Gersmann et al., 1996) did not allow detection of an AVR4-HABS. Moreover, various changes in binding conditions (pH, ionic strength, temperature- and incubation time, concentration of radiolabeled AVR4 and MFs) did not accomplish detection of an AVR4-HABS. This could possibly be due to instability or proteinase-sensitivity of the putative AVR4-HABS. However, when proteinase inhibitors were added to MF during isolation or during binding experiments, no AVR4-HABS could be detected. Alternatively, kinase activity and phosphorylation might be required for proper binding of AVR4 to its HABS, as previously demonstrated for binding of flagellin to its receptor complex (Gomez-Gomez et al., 2001). Kinase activity is also necessary for assembly of the CLAVATA complex, which consists of receptor-like protein kinase CLV1 and receptor-like protein CLV2 that regulate meristem and organ development in Arabidopsis, and for binding of this complex to CLV3 (Trotochaud et al., 2000). However, when MFs isolated from *Cf-4* tomato plants grown under contained conditions were supplied with phosphatase inhibitors or when ATP was added (with or without proteinase inhibitors) during binding assays, still no AVR4-HABS could be detected. All together, these data indicate that the AVR4-HABS is extremely unstable or that the HABS is low abundant in MFs of *Cf-4* tomato. Alternatively, binding of AVR4 to its HABS might be transient and therefore difficult to detect.

These data suggest that the mechanism of perception of AVR4 by plants carrying *Cf-4* is different from the perception of several other fungal elicitor peptides and proteins, including

AVR9, for which a HABS has been found in the plasma membrane of plants. Nevertheless, as AVR4 triggers defense responses as quickly as AVR9 (De Jong et al., 2002b), it is not conceivable that perception of AVR4 involves a low affinity-binding site. Binding of AVR4 to its binding site most likely requires additional components that were absent in our binding assays, such as soluble components present in the extracellular space of tomato leaves. This has also been proposed for recognition of AVR2 by *Cf-2* tomato, whereby AVR2 is thought to interact with Rcr3, an extracellular tomato cysteine endoprotease that is required for *Cf-2*-mediated resistance (Krüger et al., 2002). However, from our data, we cannot exclude the possibility that a direct interaction takes place between AVR4 and Cf-4. Additional experiments are required to elucidate whether AVR4 binds to Cf-4 or whether an additional component is involved, as suggested for the perception of AVR9 by plants carrying *Cf-9*.

Does circumvention of AVR recognition result in a virulence penalty for *C. fulvum*?

As production of AVRs and ECPs restricts the range of tomato genotypes that can be colonized by *C. fulvum,* the primary function of these elicitors is expected to be associated with virulence rather than with avirulence. Indeed, ECP1 and ECP2 have been demonstrated to play a role in virulence of *C. fulvum* (Laugé et al., 1997). It would therefore be beneficial for *C. fulvum* to evade induction of plant defense responses by modification of *Avr* and *Ecp* gene products in such way that their avirulence but not their virulence functions are lost.

Strains of *C. fulvum* evade *Cf-4*-mediated resistance by production of AVR4 mutant proteins carrying single amino acid substitutions that are sensitive to proteolytic breakdown, suggesting that loss of the AVR4 avirulence function is mediated by mutations that affect the conformational stability of AVR4 (Chapters 2 and 4). These AVR4 mutant proteins, however, retained the ability to bind to chitin *in vitro*, indicating that the avirulence function of AVR4 is abolished but not its putative intrinsic function in protecting the cell wall of *C. fulvum* against lysis by plant chitinases (Chapter 4). Nevertheless, a strain of *C. fulvum* virulent on plants carrying *Cf-4* was found to secrete a truncated AVR4 protein due to a frameshift mutation in the *Avr4* ORF. This strain appeared to be as virulent on susceptible tomato plants as strains that produce wild-type AVR4 (Joosten et al., 1997). Possibly, the lack of a functional AVR4 is compensated for by another protein that carries a function similar to AVR4, i.e. providing a passive protection against plant chitinases. Alternatively, as demonstrated for *P.* sojae that produces GIPs (Rose et al., 2002), this strain might have acquired more aggressive mechanisms to protect itself against plant chitinases by producing carbohydrate or protein inhibitors that bind to the active site cleft of chitinases. Indirect evidence for occurrence of

such competitive inhibitors comes from Bishop et al. (2000) who suggest that evolutionary changes in the active site cleft of plant chitinases are a direct consequence of production of inhibitors produced by fungal pathogens.

Opposed to the AVR4 mutants, the AVR4E mutant protein carrying substitutions $Phe^{62}Leu$ and $Met^{73}Thr$ ($avr4E^{LT}$) has lost its elicitor activity but is as stable as wild-type AVR4E, suggesting that *Hcr9-4E*-mediated resistance is evaded as a result of a conformational- or qualitative change in AVR4E (Chapter 2). Strains that secrete this AVR4E mutant are as virulent on susceptible tomato plants as strains that secrete wild-type AVR4E. This suggests that either AVR4E does not contribute to virulence of *C. fulvum* or both AVR4E and $avr4E^{LT}$ play a role in virulence of the fungus. In addition, the identification of only one type of modification in the *Avr4E* ORF of strains virulent on plants carrying *Hcr9-4E* suggests that mutations other than $Phe^{62}Leu$ and $Met^{73}Thr$ affect the stability of AVR4E and at the same time the virulence of *C. fulvum*. This would imply that strains identified to circumvent AVR4E recognition by lack of *Avr4E* gene expression still exhibit some level of *Avr4E* gene expression, which is below detection level, yet, high enough to contribute to virulence of *C. fulvum.*

As natural strains of *C. fulvum* that lack a functional copy of *Avr2* are as virulent as similar strains that were transformed with a functional *Avr2* gene, AVR2 appears not to be crucial for virulence of *C. fulvum* on susceptible tomato plants (Luderer et al., 2002b). AVR2, however, could potentially play a role in virulence of *C. fulvum* by suppressing the proteolytic activity of Rcr3 (Krüger et al., 2002) during growth of *C. fulvum* in the apoplastic space of tomato. Currently, efforts are being made to elucidate the role of AVR2 and Rcr3 in *Cf-2*-mediated defense responses. Also, the role of Rcr3 as a putative virulence target will be addressed.

Although AVR9 exhibits homology to a carboxypeptidase inhibitor (Van den Hooven et al., 2001), no biochemical evidence was found to prove that AVR9 is a genuine protease inhibitor. Strains of *C. fulvum* that lack the *Avr9* ORF are as virulent on susceptible tomato plants as strains that express *Avr9*, suggesting that absence of AVR9 does not have a measurable effect on virulence of the fungus. Expression of *Avr9*, however, is under control of the major nitrogen regulator NRF1 and appears to be repressed when levels of nitrate are artificially increased in the apoplast of tomato (Van den Ackerveken et al., 1992; Pérez-García et al., 2001). This suggests that AVR9 might be involved in nitrogen metabolism of *C. fulvum* and its presence might contribute to growth of *C. fulvum* under nitrogen-limiting conditions on susceptible tomato plants.

It is conceivable that no pronounced role in virulence can be assigned to single AVRs of *C. fulvum* as other proteins that exhibit a function similar to the AVRs might compensate for the

function of single AVRs, suggesting that the AVRs are redundant. In that case, AVRs might exhibit a subtle virulence function but their actual contribution to virulence might be difficult to detect under laboratory conditions. Currently, near-isogenic strains of *C. fulvum* that lack multiple *Avr* genes are created by gene silencing. It will be interesting to see whether these strains are significantly compromised in virulence on susceptible tomato plants.

Summary

Summary

Active disease resistance in plants relies on highly sensitive and specific surveillance systems that enable recognition and restriction of growth of invading pathogens. Numerous resistance (*R*) genes have been characterized from various plant species that provide typical gene-for-gene-based resistance to a variety of different pathogens that carry the matching avirulence (*Avr*) genes. Because an *Avr* gene product is not beneficial for the pathogen in the presence of the matching *R* gene, the primary function of *Avr* gene products is expected to be associated with virulence rather than with avirulence (Chapter 1). Indeed, evidence is accumulating that *Avr* genes encode effector proteins that condition establishment of a compatible interaction between a pathogen and a susceptible host, either by interacting with host-encoded virulence targets to suppress (basal) defence responses or by interfering with activities of pathogenesis-related (PR) proteins or other induced defence responses. Thus, losing an avirulence determinant, in order to overcome *R* gene-mediated resistance, might carry a virulence penalty for the pathogen.

On the introgressed region in tomato plants carrying the *Cf-4* locus (MM-Cf4 plants), five homologues of *Cladosporium fulvum* resistance gene *Cf-9* (*Hcr9-4*s) are present. In Chapter 2, we demonstrated that homologue *Hcr9-4E* confers a similar level of resistance to *C. fulvum* as homologue *Hcr9-4D*, which represents the *Cf-4* gene, through recognition of a novel avirulence determinant, designated AVR4E. The *Avr4E* gene encodes a cysteine-rich protein of 121 amino acids that is secreted into the apoplastic space of tomato as a mature protein of 101 amino acids (Chapter 2). The genomic *Avr4E* sequence of *C. fulvum* race 5 confers avirulence (after transformation) to strains of *C. fulvum* that are normally virulent on plants carrying *Hcr9-4E*, indicating that AVR4E is a genuine a race-specific avirulence determinant. Strains of *C. fulvum* evade *Hcr9-4E*-mediated resistance either by lack of *Avr4E* gene expression or by production of a stable AVR4E mutant protein that is elicitor-inactive and carries two amino acid substitutions, Phe^{62}Leu and Met^{73}Thr. Moreover, we demonstrated by site-directed mutagenesis that the single amino acid substitution Phe^{62}Leu in AVR4E is sufficient to evade *Hcr9-4E*-mediated resistance (Chapter 2).

Strains of *C. fulvum* that produce race-specific elicitor AVR4 induce a hypersensitive response (HR) leading to resistance in tomato plants carrying the *Cf-4* resistance gene. In Chapter 3, the mechanism of AVR4 perception by *Cf-4* plants is examined by performing binding studies with ^{125}I-AVR4 on microsomal membranes fractions (MFs) of MM-Cf0 (susceptible) and MM-Cf4 (resistant) tomato plants and non-host plant species. We identified an AVR4-high affinity-binding site (HABS) (K_D = 0.05 nM) that exhibited all the characteristics expected for ligand-receptor interactions, such as saturability, reversibility

and specificity. Surprisingly, the AVR4-HABS present in MFs appeared to originate from fungi present on partially infected tomato plants rather than from the tomato plant itself. This fungus-derived, AVR4-specific HABS is heat- and proteinase K-resistant, suggesting that it might be a non- proteinaceous component. Affinity cross-linking demonstrated that AVR4 specifically binds to a component of about 75 kDa of fungal origin, a phenomenon that is possibly related to the intrinsic function of AVR4 for *C. fulvum*. As no AVR4-specific HABS could be detected in MFs of tomato plants that were grown under contained conditions, the mechanism of perception of AVR4 by MM-Cf4 plants appears to be different from that found for several other fungus-derived peptides and elicitors, including AVR9, for which a HABS has been identified in MFs of several plant species.

The AVR4 protein of *C. fulvum* contains 8 cysteine residues, all of which are involved in disulfide bonds. Assignment of the disulfide bond pattern of AVR4 was partly achieved by a biological analysis using potato virus-X (PVX)-mediated expression of AVR4 disulfide bond mutants in plants carrying *Cf-4*, and subsequently confirmed and completed by partial chemical reduction of AVR4, followed by mass mapping (Chapter 4). The four disulfide bonds present in AVR4 were identified as Cys^{11}-Cys^{41}, Cys^{21}-Cys^{27}, Cys^{35}-Cys^{80} and Cys^{57}-Cys^{72}. The disulfide bond pattern and the spacing of the cysteine residues were subsequently used to carry out a motif-based search, which revealed that AVR4 contains a chitin-binding domain that is also present in chitin-binding proteins of invertebrates (inv ChBD). Three disulfide bonds in AVR4 are conserved amongst members of the inv ChBD family and these bonds are required for conformational stability of AVR4 (Chapter 4). In natural strains of *C. fulvum* disruption of two of these three conserved disulfide bonds, i.e. between Cys^{11}-Cys^{41} and Cys^{35}-Cys^{80}, results in lack of AVR4 recognition by *Cf-4* plants. However, all four disulfide bond mutants of AVR4 retained the ability to bind to chitin *in vitro.* Moreover, when bound to chitin, these disulfide bond mutants of AVR4 are less sensitive to proteolytic breakdown. Thus, while evasion of *Cf-4*-mediated resistance by *C. fulvum* appears to result from instability and protease sensitivity of AVR4 mutant proteins present in the apoplast of tomato, AVR4 mutants retained their putative intrinsic role in protecting the cell wall of *C. fulvum* against plant chitinases.

AVR4 does not require binding to chitin to induce *Cf-4*-mediated plant defence responses. In Chapter 5, we analyzed whether domains responsible for necrosis-inducing activity (NIA) and chitin-binding ability within AVR4 can be distinguished. Therefore, we performed a peptide scan (PEPSCAN) analysis using polyclonal antibodies raised against native AVR4 to identify antigenic domains containing putatively solvent-exposed residues that might condition NIA of AVR4. This antibody-affinity analysis identified one major (Cys^{41}-Cys^{57}) and two minor antigenic domains in AVR4 (Ile^{17}-Asn^{31} and Gln^{62}-Asn^{76}). Selective

alanine substitutions were performed on all, except for Cys, Pro and Gly, residues present in the major antigenic domain of AVR4, as this domain exhibits the highest affinity for AVR4 polyclonal antibodies. Moreover, to determine which residues in AVR4 are important for chitin binding, all aromatic residues and two additional residues present in conserved domains of the anticipated chitin-binding domain(s) were individually substituted by alanine. We showed that replacement of single amino acids in the major antigenic domain of AVR4 does not affect its NIA (Chapter 5). Substitution of aromatic residues Tyr^{38} and Trp^{63} by Ala, however, reduced the NIA of AVR4. However, these AVR4 mutant proteins appeared to be unstable, suggesting that Tyr^{38} and Trp^{63} contribute to conformational stability rather than to NIA of AVR4. In this study, no individual amino acid residues could be identified that are essential for binding of AVR4 to chitin, suggesting that multiple rather than single amino acid residues contribute to chitin-binding ability.

It would be beneficial for *C. fulvum* to evade induction of plant defense responses by modifying the *Avr* gene products in such way that their avirulence but not their virulence functions are lost (Chapter 6). For most single AVRs, however, no pronounced role in virulence of *C. fulvum* could be assigned, possibly due to the fact that other proteins compensated for their intrinsic functions, suggesting that AVRs are redundant.

Samenvatting

Actieve ziekteresistentie in planten is afhankelijk van buitengewoon gevoelige en specifieke controlesystemen. Deze systemen maken het mogelijk binnendringende ziekteverwekkers te herkennen, waarna de plant diverse afweermechanismen activeert, welke leiden tot een groeiremming van deze ziekteverwekkers. Een aantal resistentie (*R*) genen, afkomstig van verscheidene plantensoorten, zijn gekarakteriseerd. Deze *R* genen verschaffen de plant een typische, op het gen-om-gen principe gebaseerde, resistentie tegen ziekteverwekkers die de corresponderende avirulentie (*Avr*) genen bevatten. Gezien het feit dat in de aanwezigheid van het corresponderende *R* gen, een *Avr* gen product niet bijdraagt aan de virulentie van een ziekteverwekker, bestaat het vermoeden dat de primaire functie van *Avr* gen producten geassocieerd is met virulentie en niet met avirulentie (Hoofdstuk 1). Inderdaad, een aantal avirulentie-eiwitten blijken ziekteverwekkers in staat te stellen planten te infecteren, door een interactie aan te gaan met virulentiedoelwitten die aanwezig zijn in de gastheer en zodoende het (basale) verdedigingsmechanisme van de plant onderdrukken, of door de activiteit van zogenoemde pathogenese-gerelateerde eiwitten of andere geïnduceerde afweermechanismen te remmen. Dit houdt in dat het verlies van avirulentie-eiwitten, hetgeen mogelijkerwijs gepaard gaat met het omzeilen van *R* gen-gebaseerde resistentie, een vermindering van virulentie van de ziekteverwekker tot gevolg kan hebben.

Op het *Cf-4* locus zijn vijf *Hcr9-4*s genen aanwezig, welke vernoemd zijn naar homologen van resistentiegen *Cf-9* tegen *Cladosporium fulvum* ('*Hcr9*s'). In Hoofdstuk 2 is onderzocht of homoloog *Hcr9-4E*, door middel van herkenning van het avirulentie-eiwit AVR4E, resistentie kon geven tegen *C. fulvum* die vergelijkbaar was met die van homoloog *Hcr9-4D,* ook wel het *Cf-4* resistentiegen genoemd. Het *Avr4E* gen codeert voor een cysteïne-rijk eiwit van 121 aminozuren, dat door *C. fulvum* uitgescheiden wordt in de intercellulaire ruimtes van tomaat als een eiwit van 101 aminozuren (Hoofdstuk 2). Transformatie van virulente isolaten van *C. fulvum* met de genomische sequentie van *Avr4E* leidt tot avirulentie op planten met het *Hcr9-4E* gen. Dit houdt in dat AVR4E functioneert als een fysio-specifiek, avirulentie-eiwit. Gebleken is dat bepaalde isolaten van *C. fulvum* die virulent zijn op *Hcr9-4E* tomatenplanten het *Avr4E* gen niet tot expressie brengen, terwijl andere virulente isolaten een stabiele AVR4E mutant eiwit produceren met twee aminozuursubstituties, Phe^{62}Leu en Met^{73}Thr. In Hoofdstuk 2 laten we ook zien dat de elicitoractiviteit van AVR4E te niet wordt gedaan door de enkele aminozuursubstitutie Phe^{62}Leu, hetgeen suggereert dat deze enkele substitutie in AVR4E kan leiden tot virulentie van *C. fulvum* op *Hcr9-4E* tomatenplanten.

Isolaten van *C. fulvum* die het fysio-specifieke avirulentie-eiwit AVR4 produceren, induceren een overgevoeligheidsreactie in tomatenplanten met het *Cf-4* gen. In Hoofdstuk 3 is het mechanisme van de herkenning van AVR4 door *Cf-4* planten bestudeerd met behulp van bindingstudies met radioactief gelabeld AVR4 en microsomale membraanfracties (MFs), afkomstig van MM-Cf0 (vatbare) en MM-Cf4 (resistente) tomatenplanten en plantensoorten die geen gastheer zijn voor *C. fulvum.* Een hoge affiniteits-bindingsplaats ('HABS') voor AVR4 (K_D = 0.05 nM) werd gevonden in MFs. Deze AVR4-HABS voldeed aan alle kenmerken die verwacht kunnen worden voor ligand-receptor interacties, zoals verzadigbaarheid, reversibiliteit en specificiteit. De geïdentificeerde AVR4-HABS bleek echter niet afkomstig te zijn van de tomatenplant, maar van schimmels die aanwezig waren op gedeeltelijk geïnfecteerde tomatenplanten. Het bleek dat deze AVR4-HABS bestand was tegen hittebehandeling en tegen een behandeling met proteinase K. Dit suggereert dat de AVR4-HABS mogelijk geen eiwitachtige component is. 'Crosslink'-experimenten lieten zien dat AVR4 specifiek bindt aan een component van ongeveer 75 kDa, welke ook afkomstig is van de schimmel, hetgeen mogelijk duidt op een intrinsieke functie van AVR4 voor *C. fulvum.* In MFs, afkomstig van tomatenplanten die zijn opgegroeid in een pathogeen-vrije, geconditioneerde omgeving, is door ons geen AVR4-specifieke HABS gedetecteerd. Dit houdt mogelijkerwijs in dat het mechanisme van herkenning van AVR4 door *Cf-4* tomatenplanten verschilt van het mechanisme gevonden voor menig ander avirulentie-eiwit of peptide, inclusief AVR9, waarvoor een HABS is geïdentificeerd in MFs van verscheidene plantensoorten.

Het AVR4 avirulentie-eiwit van *C. fulvum* bezit 8 cysteine residuen, welke allen betrokken zijn bij het vormen van zwavelbruggen. Het patroon van de zwavelbruggen werd deels opgehelderd door een elicitor activiteitsanalyse uit te voeren aan AVR4 mutanten, welke door het aardappelvirus X ('PVX') tot expressie werden gebracht in *Cf-4* tomatenplanten (Hoofdstuk 4). Ook werd er gebruik gemaakt van een chemische methode, waarbij de massa van verschillende AVR4 fragmenten, welke werden verkregen na digestie van gedeeltelijk gereduceerd AVR4 eiwit, werd bepaald. De vier zwavelbruggen aanwezig in AVR4 werden geïdentificeerd als zijnde Cys^{11}-Cys^{41}, Cys^{21}-Cys^{27}, Cys^{35}-Cys^{80} en Cys^{57}-Cys^{72} (Hoofdstuk 4). Vervolgens werd er met behulp van het patroon van de zwavelbruggen en de onderlinge afstand van de cysteine residuen gezocht naar bekende sequentiemotieven in AVR4. Een chitine-bindend domein werd gevonden in AVR4 dat sterk lijkt op het chitine-bindend domein van invertebraten ('inv ChBD'). Gebleken is dat de drie geconserveerde zwavelbruggen in AVR4, met uitzondering van Cys^{21}-Cys^{27}, ook essentieel zijn voor de stabiliteit van AVR4 (Hoofdstuk 4). In natuurlijke isolaten van *C. fulvum* wordt de herkenning door *Cf-4* planten omzeild door verbreking van de Cys^{11}-Cys^{41} of Cys^{35}-Cys^{80}

zwavelbrug. Gebleken is dat AVR4 nog steeds chitine kan binden wanneer één zwavelbrug verbroken is. Ook zijn deze zwavelbrugmutanten minder gevoelig zijn voor proteolytische afbraak wanneer ze gebonden zijn aan chitine. Dus, hoewel de productie van instabiele AVR4 mutanten tot gevolg heeft dat isolaten van *C. fulvum* virulent worden op *Cf-4* tomatenplanten, hebben deze mutanten hun vermeende intrinsieke functie, namelijk het beschermen van de celwand van *C. fulvum* tegen chitinases, behouden.

Binding van AVR4 aan chitine is niet vereist om een afweer te induceren in *Cf-4* tomatenplanten. In Hoofdstuk 5 is onderzocht of er onderscheid gemaakt kan worden tussen domeinen die verantwoordelijk zijn voor de necrose-inducerende activiteit ('NIA') van AVR4 en domeinen die betrokken zijn bij de binding van AVR4 aan chitine. Om in AVR4 antigene domeinen te identificeren die mogelijk betrokken zijn bij de NIA van AVR4, is er een affiniteitsanalyse uitgevoerd met synthetische, overlappende peptiden van AVR4 ('PEPSCAN') en polyklonaal serum gemaakt tegen natief AVR4. Deze analyse resulteerde in de identificatie van één groot (Cys^{41}-Cys^{57}) en twee kleinere (Ile^{17}-Asn^{31} and Gln^{62}-Asn^{76}) antigene domeinen in AVR4 (Hoofdstuk 5). Daar het grote antigene domein in AVR4 de hoogste affiniteit heeft voor polyklonale AVR4 antilichamen, zijn alle residuen, behalve Cys, Pro, and Gly, aanwezig in dit domein, vervangen door alanine en is de NIA van de mutanten bepaald. Ook is gekeken naar residuen in AVR4 die mogelijk belangrijk zijn voor chitine binding door alle aromatische residuen en twee additionele residuen, die geconserveerd zijn in mogelijke chitine-bindende domeinen, afzonderlijk te vervangen door Ala. Substitutie van de afzonderlijke aminozuren in het grote antigene domein van AVR4 leidde niet tot een significante verandering van de NIA van AVR4 (Hoofdstuk 5). Echter, substitutie van de aromatische residuen Tyr^{38} and Trp^{63} door Ala reduceerde de NIA van AVR4 aanzienlijk. Het bleek echter dat deze AVR4 mutanten niet stabiel waren, hetgeen betekent dat Tyr^{38} and Trp^{63} bijdragen aan de stabiliteit maar niet aan de NIA van AVR4. De afzonderlijke aminozuursubstituties in deze studie hadden in geen van de gevallen effect op het chitine-bindende vermogen van AVR4. Mogelijk zijn hier meerdere residuen bij betrokken en is er een veelvoud aan substituties nodig om een reductie teweeg te brengen in het chitine-bindende vermogen van AVR4.

Het zal voor *C. fulvum* waarschijnlijk heilzaam zijn om avirulentie-eiwitten zodanig te modificeren dat deze niet langer herkend worden, maar nog wel kunnen bijdragen aan virulentie (Hoofdstuk 6). Aan de meeste avirulentie-eiwitten van *C. fulvum* kon tot dusver echter nog geen duidelijke rol in virulentie worden toegeschreven. Waarschijnlijk kan de functie van een groot deel van deze avirulentie-eiwitten overgenomen worden door andere eiwitten, hetgeen betekent dat de functie van deze avirulentie-eiwitten uitwisselbaar is.

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Nawoord

Nu alleen nog de verdediging, en dan…KLAAR! Na 5 jaar zwoegen en zweten is er dan toch een einde gekomen aan dit project, met als trots en tevreden resultaat: Dr. Nienk! De afgelopen periode is niet alleen een hele gezellige maar ook een erg leerzame periode geweest. Zonder de hulp van mijn familie, vrienden, en collega's zou dit boekje niet tot stand zijn gekomen! Ik wil dan ook als eerste trouwe Jopie, bolle Gijs (die altijd over mijn toetsenbord liep) en wijlen Heinzje (mooiste mannetje van Utrecht) bedanken voor hun afleiding tijdens het vele thuiswerken.

Papa en mama, hier is die dan: mijn boekje! Mama, op cruciale momenten zorgde jij voor het juiste perspectief. Jij hebt mij geleerd wat echt belangrijk is! Papa, jou heb ik vele malen om advies gevraagd en altijd gaf jouw inzicht een verheldering van de situatie. Fijn dat je mij ter zijde staat (zit) als een van mijn paranimfen tijdens de verdediging van mijn proefschrift. Bedankt lieve ouders, dat jullie altijd voor mij klaar hebben gestaan.

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Matthieu, de verhuizing van alle Clado's naar het eerste Clado-lab gaf jou de mogelijkheid om je te bemoeien met mij en mijn onderzoek. Ik moet zeggen: dit heeft mij goed gedaan! Jouw betrokkenheid (o.a. "proefjes doen in het lab") heeft een belangrijke bijdrage geleverd aan het tot stand komen van mijn proefschrift. Ook wil ik je bedanken voor je geduld en tijd die je gestoken hebt in het (nauwkeurige!) nakijken van mijn hoofdstukken. Veel geluk gewenst met de uitbreiding van je gezin!

En dan hebben we natuurlijk de Clado's! Bench-maatje John, met jou heb ik veel lol gehad. Misschien iets teveel, want in het tweede Clado-Lab wilde je niet meer naast me zitten (te onrustig tijdens het inzetten van een "PCR reactie" ??). Boterhammen met nutella/pindakaas, wenkbrauwen die BD spreken, sandalen: Je kan niet vroeg genoeg beginnen met afbouwen…..Wie weet, kom ik nog eens langs (…). Kamergenootje Marco, zingen kun je als de beste hoor. Ik heb het altijd erg gezellig gevonden op onze kamer. Bas, zonder jou geen Hoofdstuk 2. Bedankt voor je input! Misschien wil John wel een keer gebruik maken van het logeerbed (weet jij nog hoe groot de voordeur was bij "Onder de

Linden"?). Renier, als er één iemand in de wetenschap thuis hoort, ben jij dat wel. Vele discussies hebben we gehad en vaak heb je me geholpen bij nieuwe experimenten. Bedankt! Paranimf Camiel (Camilio), vaak heb je het (achter de schermen) voor mij opgenomen en meerdere keren had ik dat niet eens in de gaten...Dabberen kan je als de beste en altijd oog voor (mooie) meisjes. Ik hoop dat je snel een uitdaging vindt waarin je al je wilde ideeën kwijt kan. Rianne, jij voelde goed aan wanneer het mij teveel werd en zorgde voor leuke afleiding. Bedankt! Maarten, bij plantenveredeling helemaal je draai gevonden (de "overkant" houdt je in de gaten hoor!). Suzanne, altijd in de weer. Ik hoop dat deze ijverigheid beloond gaat worden met leuke resultaten. Harrold, hoewel onze samenwerking enigszins onverwacht tot stand kwam, heb ik plezierig met je samengewerkt. Rob, ik heb altijd erg kunnen genieten van je absurde en wilde verhalen. Ik ken niemand die zoveel (gekke) dingen heeft mee gemaakt als jij! Ik hoop dat we elkaar in de toekomst blijven zien. Iris, ik heb jou met veel plezier begeleid toen je nog student was en ik wil je bedanken voor je bijdrage aan mijn proefschrift (het is toch een hoofdstukje geworden!). Paul, ik heb nu eenmaal nog nooit iemand gezien met zo veel borsthaar. Frank, ik wil je bedanken voor het brainstormen en je wetenschappelijke input tijdens werkbesprekingen. Jouw enthousiasme in het Lab, en in de trein (...), hebben we allemaal denk ik wel een beetje gemist. Richard, I enjoyed the many fruitful discussions we had (especially in Loburg), and I look forward to see you on the 5th of March.

En niet te vergeten: alle niet-Clado's. Wilco, dansen tot je erbij neervalt. Jan, jammer dat je er niet bij kan zijn. Arjen, (bijna?) overal verstand van. Anna, falling on the floor while dancing doesn't hurt. Peter, eet niet te veel patat. Maurice, leukste student bij Fyto. Martijn, lekker hard lachen is goed! Ali en Ria, voor al uw administratieve problemen. Wim, jij had alles in de kast. Ioannis, hope things work out in future (no army for you?). Lute-Harm, Jos en Sander; altijd gezellig tijdens koffiepauzes. Marcel, bedankt voor je steun! Maïta, succes met de afronding en veel geluk gewenst in Engeland. Ook wil ik alle andere collega's die ik hier niet genoemd heb bedanken voor de gezelligheid tijdens koffiepauzes, labuitjes, borrels (!) en (cacao) kerstdiners.

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Dave, drinking without eating: will we ever learn. I enjoyed spending time with you in Wageningen (especially in Loburg, Vlaamsche Reus and “De Zaaier”), on Xanthos, in France (camping) and on skiing holidays.... I think you made an excellent decision by moving to Utrecht and I hope we keep in touch in future.

En dan zijn er natuurlijk mijn lieve broertjes, Peter en Jeroen. Ook jullie hebben alles van dichtbij mee mogen maken. Het is inderdaad eindelijk af. Peet, jou (nuchtere) opvattingen hebben mij de afgelopen jaren enorm gesteund. Ook hebben wij vele gezellige avonden gehad (waarvan meerdere in ’t Hart) en ik hoop dat er nog meer zullen volgen. Jeroenie (ook wel Sabaronni), bij jou heb ik vaak mijn hart kunnen luchten en ik wil je bedanken voor je inlevingsgevoel. Ik kijk met erg veel plezier terug op onze vakanties samen en hoop dat we nog vaak samen zullen fietsen.

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Curriculum vitae

Nienke Westerink werd op 22 mei 1974 geboren in Groningen. In juni 1992 behaalde zij aan de Menso Alting College in Hoogeveen het V.W.O. diploma. In datzelfde jaar begon zij aan haar studie biologie aan de Universiteit van Utrecht, met als specialisatierichting Moleculaire Microbiologie. De specialisatiefase omvatte twee stageperiodes, beide van ongeveer 9 maanden. Bij de vakgroep Moleculaire Microbiologie aan de Universiteit van Utrecht heeft zij onderzoek verricht aan de vouwing van het buitenmembraan eiwit PhoE van *Escherichia coli* onder begeleiding van Dr. E. F. Eppens, Dr. J. P. M. Tommassen en Prof. dr. W. P. M. Hoekstra. Bij de sectie Fytopathologie van het voormalige biotechnologische bedrijf MOGEN in Leiden heeft zij onderzoek verricht aan de complementaire eigenschappen van verschillende chitinases die aanwezig zijn in planten. Zij vervolgde deze stage met een tijdelijke aanstelling als onderzoeker bij de sectie Nematologie van MOGEN. In mei 1997 behaalde zij haar doctoraal diploma en in datzelfde jaar begon zij haar promotieonderzoek bij het laboratorium voor Fytopathologie aan de Universiteit van Wageningen onder begeleiding van Dr. ir. M.H.A.J. Joosten en Prof. dr. ir. P.J.G.M. de Wit. Het promotieonderzoek werd ondersteund door een onderzoeksbeurs van de Universiteit van Wageningen en de resultaten van dit onderzoek staan beschreven in dit proefschrift.

List of publications

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