The *Cladosporium fulvum*-tomato interaction: elicitor proteins and their perception

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Stellingen

1. Alhoewel avirulentiegenen meestal gekloneerd worden op basis van hun rol bij avirulentie van het pathogeen zal dit niet hun intrinsieke functie zijn.
   Gabriel, D.W. (1999) Why do pathogens carry avirulence genes? Physiol. Mol. Plant Pathol. 55, 205-214.
   Van 't Slot, K.A.E. and Knogge, W. (2002) A dual role of microbial pathogen-derived proteins in plant disease and resistance. Crit. Rev. Plant Sci., in press.

2. Het functioneren van avirulentie-eiwitten kan zowel bij virulentie als bij avirulentie van het pathogeen gebaseerd zijn op interactie met het virulentiedoelwit.
   Van der Biezen, E. and Jones, J.D.G. (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. 23, 454-456.
   Dit proefschrift.

3. Perceptie van avirulentie-eiwitten van een pathogeen door resistentie-eiwitten van de gastheer, lijkt overwegend indirect te zijn.
   Dit proefschrift.

4. De betrokkenheid van meerdere planteneiwitten bij de specifieke herkenning van een avirulentie-eiwit vereist behoedzaamheid bij het gebruik van het begrip resistentiegen.
   Van der Biezen, E. and Jones, J.D.G. (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. 23, 454-456.
   Dit proefschrift.

5. Bij indirecte perceptie zou uitbreiding van het twee-componenten systeem tot een drie-componenten systeem de toepassingsmogelijkheden in de moleculaire resistentie-veredeling aanzienlijk kunnen verruimen.
   De Wit, P.J.G.M. (1992) Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. Annu. Rev. Phytopathol. 30, 391-418.

6. De kunst van het laveren is om zo recht mogelijk op je doel af te gaan zonder de vaart te verliezen.

7. De uiteindelijke invoering van een uniform e-mailadres voor medewerkers van Wageningen UR duidt op een traag, maar voortschrijdend inzicht in de functie van e-mail.
   Weekblad voor Wageningen UR 3, 2002.

8. Opruimen loont de moeite!

Stellingen behorende bij het proefschrift:
'The *Cladosporium fulvum*-tomato interaction: elicitor proteins and their perception'
door Rianne Luderer, Wageningen, 29 mei 2002

Voor mijn zusje

Contents

Chapter 1

General introduction and outline of the thesis

Plant-pathogen interactions

Plants are constantly exposed to pathogens that are present in their environment. In most cases, plants do not get diseased, because the pathogen lacks the basic capacity to infect the plant species. Such a lack of basic compatibility between a plant species and a pathogen is called nonhost resistance (Agrios, 1997). In only a few cases, basic compatibility between a particular plant species and an encountering pathogen exists. In principle, the pathogen is able to colonise and reproduce successfully on that particular plant species under certain conditions. However, in most cases in nature, some genotypes of the susceptible plant species are able to recognise the invading pathogen and arrest its development, resulting in host resistance and avirulence of the pathogen. This phenomenon is referred to as genotype-specific resistance. Genetic studies revealed that genotype-specific resistance is generally depending on a monogenic, dominant trait. In addition, avirulence of the pathogen was shown to be dependent of a monogenic, dominant trait as well. Based on these findings the gene-for-gene concept was put forward.

The gene-for-gene concept

The gene-for-gene concept was independently proposed by Oort (1944), working on the *Ustilago tritici*-wheat interaction and by Flor (1942, 1946), working on the *Melampsora lini*-flax interaction. Based on their genetic studies they postulated that for every dominant gene determining resistance in the host plant, there is a matching dominant gene conditioning avirulence in the pathogen. The prevailing model to explain the biochemical basis of the gene-for-gene concept is the elicitor-receptor model (Gabriel and Rolfe, 1990; Keen, 1990). According to this model, an avirulence (*Avr*) gene of a pathogen encodes an elicitor protein that is perceived by a receptor protein, which is encoded by the matching resistance (*R*) gene of the host plant. Perception of the elicitor protein by the host plant subsequently leads to the activation of defence responses, often including local cell death around the infection site (Hammond-Kosack and Jones, 1996). The latter is referred to as the hypersensitive response (HR) and is a hallmark for gene-for-gene based resistance.

Resistance gene and avirulence gene products

To date, a variety of *R* and *Avr* genes have been cloned (reviewed by Takken and Joosten, 2000a and Van 't Slot and Knogge, 2002). Various *R* genes have been characterised that confer resistance to a wide range of pathogens. The encoded R proteins, however, are remarkably similar and can be classified based on their structural domains and their localisation (Takken and Joosten, 2000a). Consistent with their predicted receptor function all R proteins, except for Pto and RPW8, contain a leucine-rich repeat (LRR) domain, which is thought to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). The *R* genes can be divided in those encoding proteins with cytoplasmic LRRs and those encoding proteins with extracytoplasmic LRRs (Jones and Jones, 1996). The R

proteins with a cytoplasmic LRR domain all possess a nucleotide-binding site (NBS). Furthermore, some of these R proteins contain a leucine zipper (LZ) domain or a Toll/interleukin 1 receptor-like (TIR) domain. The R proteins with an extracytoplasmic LRR domain contain a transmembrane region and one of them, Xa21, contains a cytoplasmic protein kinase domain as well (Takken and Joosten, 2000a). The structural domains of R proteins predict a role in signal perception and transduction. In most cases, however, it is not clear how R proteins initiate defence responses.

In contrast to R proteins, *Avr* gene products identified so far show hardly any similarity. The fact that *Avr* genes are maintained within a pathogen population suggests that their products, in addition to a role as avirulence factor, have a function that is beneficial for the pathogen. Although the number of *Avr* genes for which a clear function for the pathogen has been demonstrated is still limited, it is now generally accepted that *Avr* gene products are bifunctional. In addition to triggering defence responses on resistant host genotypes, they have a role in virulence of the pathogen on susceptible genotypes (Gabriel, 1999; White *et al.*, 2000; Kjemtrup *et al.*, 2000; Van 't Slot and Knogge, 2002).

Perception of AVR proteins

The number of *R* genes for which the matching *Avr* gene has been cloned is increasing. However, to date, only for two R proteins, Pto from tomato and Pi-ta from rice, physical interaction with a pathogen-derived ligand, AvrPto and AVR-Pita respectively, has been reported (Scofield *et al.*, 1996; Tang *et al.*, 1996; Jia *et al.*, 2000). This limited number suggests that in various host-pathogen relationships no physical interaction between R and AVR proteins occurs and that perception of AVR proteins by their matching R gene products is indirect. Indirect perception of AVR proteins by R proteins implies that at least a third component is required for specific recognition of an avirulence factor by a resistant host. Although the nature of the third component is speculative, it might well be a co-receptor. Another fascinating possibility was put forward by Van der Biezen and Jones (1998), in the 'Guard hypothesis'. The 'Guard hypothesis' proposes that the third component that is required for perception of an AVR protein is represented by the virulence target of the AVR protein. Binding of the AVR protein to its virulence target is detected by the matching R protein, which is 'guarding' this virulence target. An intriguing aspect of the 'Guard hypothesis' is that it predicts that, in plants carrying the matching *R* gene, the *Avr* gene product causes avirulence by interaction with its virulence target. This would mean that, although AVR proteins are generally regarded to be bifunctional, this dual function might be based on a single biochemical event, funnelling into two different pathways. In the presence of the 'guarding' R protein, binding of the AVR protein to its virulence target will result in the initiation of defence responses, whereas in case of absence of the R protein, binding will result in enhanced susceptibility of the host.

The *Cladosporium fulvum*-tomato interaction

The fungus *Cladosporium fulvum* (syn. *Fulvia fulva*) is a biotrophic pathogen that causes leaf mould disease on its only host, tomato (*Lycopersicon spp.*). Conidia of *C. fulvum* germinate on the leaf surface and enter the leaf through the stomata. Subsequently, the hyphae colonise the intercellular space of the leaves of a susceptible host. Although the hyphae grow in close contact with the mesophyll cells, no specialised feeding structures like haustoria are formed (De Wit, 1977). Approximately 10 days after penetration, a network of hyphae is formed in the substomatal cavities, and conidiophores on which asexual spores are formed, emerge from the stomata.

The interaction between tomato and *C. fulvum* complies with the gene-for-gene concept. As growth of *C. fulvum* is confined to the apoplastic space of tomato leaves, it was hypothesised that exchange of all molecules between plant and fungus, including elicitor proteins, occurs via the apoplastic space. De Wit and Spikman (1982) indeed showed that elicitor molecules are present in apoplastic fluid of infected susceptible plants. Two race-specific elicitors, AVR4 and AVR9, were isolated from apoplastic fluid and the corresponding genes were cloned (Joosten *et al.*, 1994; Scholtens-Toma and De Wit, 1988; Van den Ackerveken *et al.*, 1992; Van Kan *et al.*, 1991). Furthermore, five elicitor proteins, extracellular proteins (ECPs) ECP1, ECP2, ECP3, ECP4 and ECP5, were purified that are produced by all strains of *C. fulvum* analysed so far (Laugé *et al.*, 2000; Laugé *et al.*, 1998b; Van den Ackerveken *et al.*, 1993a; Wubben *et al.*, 1994). For ECP1 and ECP2 it has been shown that they are virulence factors of *C. fulvum* (Laugé *et al.*, 1997) and it is expected that the same holds for the other ECP proteins. All elicitor proteins of *C. fulvum* that have been isolated so far, are small, cysteine-rich proteins that contain an N-terminal signal sequence for extracellular targeting (reviewed by Joosten and De Wit, 1999).

From tomato, several *Cf* genes mediating resistance to specific races of *C. fulvum* have been cloned (reviewed by Joosten and De Wit, 1999). Two of them, *Cf-4* and *Cf-9* (Jones *et al.*, 1994; Thomas *et al.*, 1997) confer resistance upon recognition of elicitor proteins AVR4 and AVR9, respectively. All *Cf* genes are predicted to encode extracytoplasmic, membrane-anchored glycoproteins that contain many LRRs. The N-terminal part of the LRR domain, which is highly variable among the Cf proteins, is thought to play a role in recognitional specificity, whereas the C-terminal part of the LRR domains of Cf proteins is highly conserved and might play a role in signal transduction (Dixon *et al.*, 1996).

Outline of the thesis

The research described in this thesis is focussed on the characterisation of elicitor proteins of *C. fulvum* and their perception by resistant tomato plants. At the start of this research project several *Cf* genes from tomato, mediating resistance to specific races of *C. fulvum*, and several *Avr* and *Ecp* genes from *C. fulvum* had already been cloned. The apoplastic localisation of the various elicitor proteins, together with the predicted extracellular

localisation of the LRR region of the Cf proteins is consistent with the hypothesis that a direct interaction occurs between *C. fulvum*-derived elicitors and the matching Cf proteins. However, no biochemical data were available to confirm this.

A striking feature of all elicitor proteins of *C. fulvum* is that the mature form contains an even number of cysteine residues (reviewed by Joosten and De Wit, 1999). These cysteine residues are thought to be involved in disulfide bridges, which are essential for proper conformation and stability of the elicitors. Therefore we set out to examine the role of the cysteine residues in HR-inducing activity and stability of elicitor proteins. Chapter 2 describes a study in which a mutational analysis of the cysteine residues of elicitor proteins ECP1, ECP2 and ECP5 was performed. Our findings indicate that the role of (the even number of) cysteine residues in these proteins is more complex than anticipated, as some of the cysteine residues do not seem to be involved in intramolecular disulfide bridges in the mature ECPs.

The six cysteine residues present in the mature AVR9 peptide have been shown to be involved in intramolecular disulfide bridges that are essential for its structure and necrosis-inducing activity (Kooman-Gersmann *et al.*, 1997; Van den Hooven *et al.*, 2001). Binding studies using radiolabeled AVR9 protein showed the presence of a high-affinity binding site (HABS) for this elicitor in plasma membranes isolated from leaves of near-isogenic lines of tomato, either with or without the *Cf-9* resistance gene (Kooman-Gersmann *et al.*, 1996). This raised the question whether, in addition to binding of AVR9 to the HABS, a direct interaction between AVR9 and the Cf-9 protein does take place. In order to perform binding studies with Cf-9 protein in the absence of the HABS, Cf-9 was produced in heterologous systems. In chapter 3 the results of binding studies with Cf-9 protein produced in insect cells or other heterologous systems are described. Although binding studies were performed using different experimental approaches, no specific binding of AVR9 to Cf-9 was detected. This implies that the simplest interpretation of the gene-for-gene concept, involving direct interaction of a pathogen-derived elicitor with a matching resistance gene product, does not hold for the *Avr9/Cf-9* gene pair, and that at least a third interacting partner is involved in perception of AVR9 by Cf-9. The HABS would be a good candidate for this third interacting partner. Currently, efforts are being made to purify the HABS.

Avr2/Cf-2 is another gene-for-gene pair for which a third interacting partner has been suggested to play a role in perception of the elicitor protein by tomato (Dixon *et al.*, 2000). The gene encoding the possible third interacting partner, *Rcr3*, was identified and its cloning is underway. *Rcr3* is specifically required for *Cf-2*-mediated resistance. The *Cf-2* resistance locus has been isolated (Dixon *et al.*, 1996), however, cloning of the *Avr2* gene has been unsuccessful so far, as attempts to purify the AVR2 protein from apoplastic fluid failed. To allow dissection of the biochemical mechanism of perception of AVR2 by Cf-2 and Rcr3, we set out to clone *Avr2* cDNA, employing a functional screen based on the HR-inducing activity of AVR2 in Cf2 tomato plants (Takken *et al.*, 2000b). In chapter 4, cloning and characterisation of avirulence gene *Avr2* is described. Whether Rcr3 is indeed involved in perception of AVR2 by Cf-2 will be investigated in the near future.

In chapter 5, the perception of AVR proteins is discussed for gene-for-gene relationships for which both the *Avr* and the matching *R* gene have been cloned. Interestingly, for a majority of the gene-for-gene relationships, indirect perception of AVR proteins seems to be more consistent with the experimental data than a direct AVR-R protein interaction. Moreover, this indirect perception seems to comply with the 'guard' model. The implications of this finding are discussed.

The data presented in this thesis indicate that, although the genetics of the gene-for-gene concept suggest a simple direct receptor-ligand interaction, the actual biochemical mechanism of elicitor perception by a resistant host might be more complex than anticipated. In chapter 6, models are presented for perception of AVR2 and AVR9 by Cf2 and Cf9 tomato plants, respectively. The implications of indirect perception of AVR proteins by resistant host plants for future research on gene-for-gene interactions and for modern resistance breeding will be discussed.

Chapter 2

Functional analysis of cysteine residues of ECP elicitor proteins of the fungal tomato pathogen *Cladosporium fulvum*

This chapter has been published with minor modifications by Rianne Luderer, Maarten J.D. de Kock, Robert H.L. Dees, Pierre J.G.M. de Wit and Matthieu H.A.J. Joosten in Molecular Plant Pathology 3 (2), 91-95 (2002).

Abstract

A striking feature of all elicitor proteins of *Cladosporium fulvum* that are specifically recognised by tomato is that they contain an even number of cysteine residues. These cysteine residues are thought to be involved in disulfide bridges. In this study, a mutational analysis of the cysteine residues of ECP1, ECP2 and ECP5 was performed, to examine their role in stability and hypersensitive response-inducing activity of the proteins. We show that not all cysteine residues of the ECPs are critical for the hypersensitive response-inducing activity of the proteins and we propose that the role of cysteine residues in the ECPs is more complex than anticipated.

Introduction

The interaction between tomato and the strictly apoplastic, biotrophic pathogen *Cladosporium fulvum* complies with the gene-for-gene model. This model postulates that for every gene determining resistance in the host, there is a corresponding gene conditioning avirulence in the pathogen (Flor, 1942). Avirulence proteins of *C. fulvum* elicit a hypersensitive response (HR) in tomato, resulting in resistance. To date, 8 genes from *C. fulvum* have been cloned that encode an elicitor protein. Four of these elicitors, AVR2, AVR4, AVR4E and AVR9, are race-specific and induce a HR, visible as necrosis, following injection in tomato lines harbouring the corresponding *C. fulvum* (*Cf*) resistance gene (Luderer, unpublished data; Joosten *et al.*, 1994; Westerink, unpublished data; Scholtens-Toma and De Wit, 1988). The other four elicitors, extracellular proteins ECP1, ECP2, ECP4 and ECP5, are produced by all strains of *C. fulvum* investigated thus far and are recognised by genotypes of different *Lycopersicon* species (Haanstra *et al.*, 2000; Laugé *et al.*, 2000). ECP1 and ECP2 have been proven to be virulence factors of *C. fulvum* (Laugé *et al.*, 1997) and it is expected that the same holds true for ECP4 and ECP5. None of the elicitors have a significant homology with each other or with any other genes or proteins present in databases.

During infection, *C. fulvum* secretes the elicitor proteins into the apoplast of tomato leaves. Consistent with this observation, the proteins all contain an N-terminal signal sequence for extracellular targeting (Joosten *et al.*, 1994; Laugé *et al.*, 2000; Luderer, unpublished data; Van den Ackerveken *et al.*, 1993a; Van Kan *et al.*, 1991; Westerink, unpublished data). After secretion, additional N- and/or C-terminal processing takes place (Joosten *et al.*, 1997; Laugé *et al.*, 2000; Van den Ackerveken *et al.*, 1993a,b). A striking feature of all elicitor proteins is that the mature form contains an even number of cysteine residues. The six cysteine residues present in the mature AVR9 peptide have been shown to be involved in disulfide bridges that are essential for its structure and necrosis-inducing activity (Kooman-Gersmann *et al.*, 1997; Van den Hooven *et al.*, 2001). Furthermore, in several natural mutant isoforms of AVR4, produced by strains of *C. fulvum* that are virulent on tomato plants that carry the *Cf-4* gene, one of the eight cysteine residues is substituted

by a tyrosine residue, resulting in an unstable protein (Joosten *et al.*, 1997). These data suggest that the various cysteine residues that are present in elicitor proteins of *C. fulvum* are involved in disulfide bridges, which are essential for proper conformation and stability of the elicitors. The stability of the elicitor proteins might be particularly important because they reside in the apoplast, which is known to be rich in proteases (Van den Ackerveken *et al.*, 1993b). The stability of ECPs in the apoplast is thought to be required both for their contribution to the virulence of the fungus and for their elicitor function. To find out whether the even amount of cysteine residues is of general importance for the stability and necrosis-inducing activity of the various elicitor proteins of *C. fulvum*, a mutational analysis was initiated.

Results and conclusions

In this study, cysteine residues of ECP5, ECP1 and ECP2, containing 6, 8 and 4 cysteine residues, respectively (Fig. 1), were substituted one by one by an alanine residue, using primer-directed mutagenesis of the corresponding codon of the encoding cDNA. Wild-type *Ecp* cDNAs and derived mutants were expressed in tomato genotypes carrying the corresponding *Cf-ECP* gene (Laugé et al., 1998b, 2000), using potato virus X (PVX). Previously, the PVX expression system was shown to be well suited for testing HR-inducing activity of AVR9 mutants (Kooman-Gersmann *et al.*, 1997). For each ECP and its derivatives, the HR-inducing activity was classified ranging from no HR-inducing activity (-), and showing only mosaic symptoms caused by PVX, to the HR-inducing activity similar to that of the wild-type ECP (+++++). In Figure 2a-c, typical HR symptoms, consisting of spreading necrotic lesions and epinasty of leaves, are shown for each of the wild type ECPs and some of their derivatives.

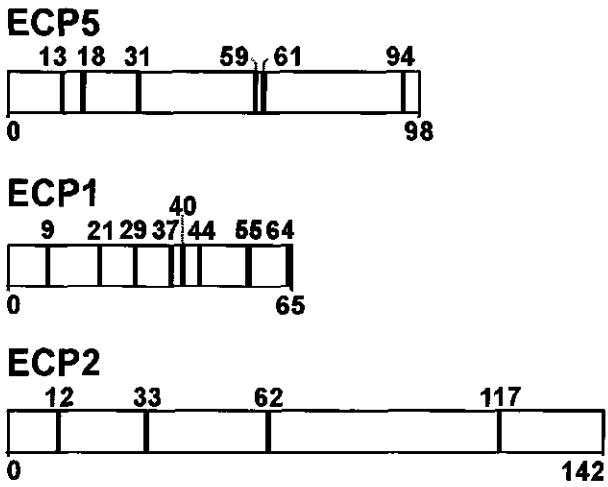

Fig. 1. Schematic representation of mature ECP5, ECP1 and ECP2. Numbers indicate amino acid positions in the mature proteins. The black bars indicate the positions of the cysteine residues.

Single cysteine-to-alanine substitutions in ECP5 resulted in a complete loss of necrosis-inducing activity for residues 31, 59, 61 and 94 (see Fig. 2a for symptoms of C31A), suggesting that these are involved in disulfide bridges which are essential for HR-inducing activity and/or stability of the ECP5 protein. Wild-type HR-inducing activity was observed for C13A and C18A derivatives (see Fig. 2a for symptoms of C13A), indicating that cysteine residues 13 and 18 are either not connected by a disulfide bridge or that the disulfide bridge that they form is not essential for the necrosis-inducing activity and stability of the ECP5 protein. It was not possible to analyse the stability of ECP5 and derived proteins by Western blot analysis, as attempts to raise antibodies against a synthetic peptide derived from ECP5 were not successful.

Single cysteine-to-alanine substitutions in ECP1 resulted in a complete loss of HR-inducing activity for all mutants, except for C64A, which showed a reduced HR-inducing activity (+++) compared to the wild-type ECP1 protein (Fig. 2b,d). For two cysteine residues that are connected by an intramolecular disulfide bridge, a similar effect on the HR-inducing activity of the protein is expected following substitution of each of the matching cysteine residues by alanine. Therefore, it is surprising that only one mutant shows some residual HR-inducing activity, and a matching mutant is lacking. These results indicate that C64, and consequently also one additional cysteine residue, are not involved in an intramolecular disulfide bridge in the mature ECP1 protein. These cysteine residues might be involved in disulfide bridges that are required in intermediate structures during folding of the protein, or in intermolecular disulfide bridges. Western blot analysis (see legend of Fig. 2) shows that there is a strict correlation between the amount of ECP1 and its derivatives that is present in the apoplastic fluid, and their HR-inducing activity (Fig. 2d), suggesting that the changes in HR-inducing activity are due to changes in the stability of the ECP1 mutants.

Substitution of cysteine residue 12, 33 or 62 of ECP2 by alanine resulted in a reduction of HR-inducing activity (+++) compared to wild-type ECP2, whereas substitution of C117 resulted in an almost complete loss of HR-inducing activity (+) (Fig. 2c,e). As substitution of none of the other cysteine residues resulted in a loss of HR-inducing activity similar to the C117A mutant, C117 and at least one other cysteine residue are probably not involved in an intramolecular disulfide bridge in the mature ECP2 protein. To find out whether the other cysteine residues are involved in an intramolecular disulfide bridge, double mutants of *Ecp2* were constructed. Only C33,62A showed an HR-inducing activity, which was equal to the activity of the corresponding single mutants (Fig. 2e), indicating that these cysteine residues are involved in an intramolecular disulfide bridge. Western blot analysis (see legend of Fig. 2) revealed that there is not a strict correlation between the amount of ECP2 and derived proteins that is present in the apoplastic fluid, and their HR-inducing activity, as C117A is readily detected by ECP2 antibodies, whereas hardly any HR-inducing activity was observed (Fig. 2e). In particular, C117A shows a changed pattern on Western blot compared to the wild-type ECP2 protein. This might be the result of structural differences, due to aberrant folding or the potential loss of the ability to form intermolecular disulfide bridges, which result in a different behaviour of the protein on a native gel.

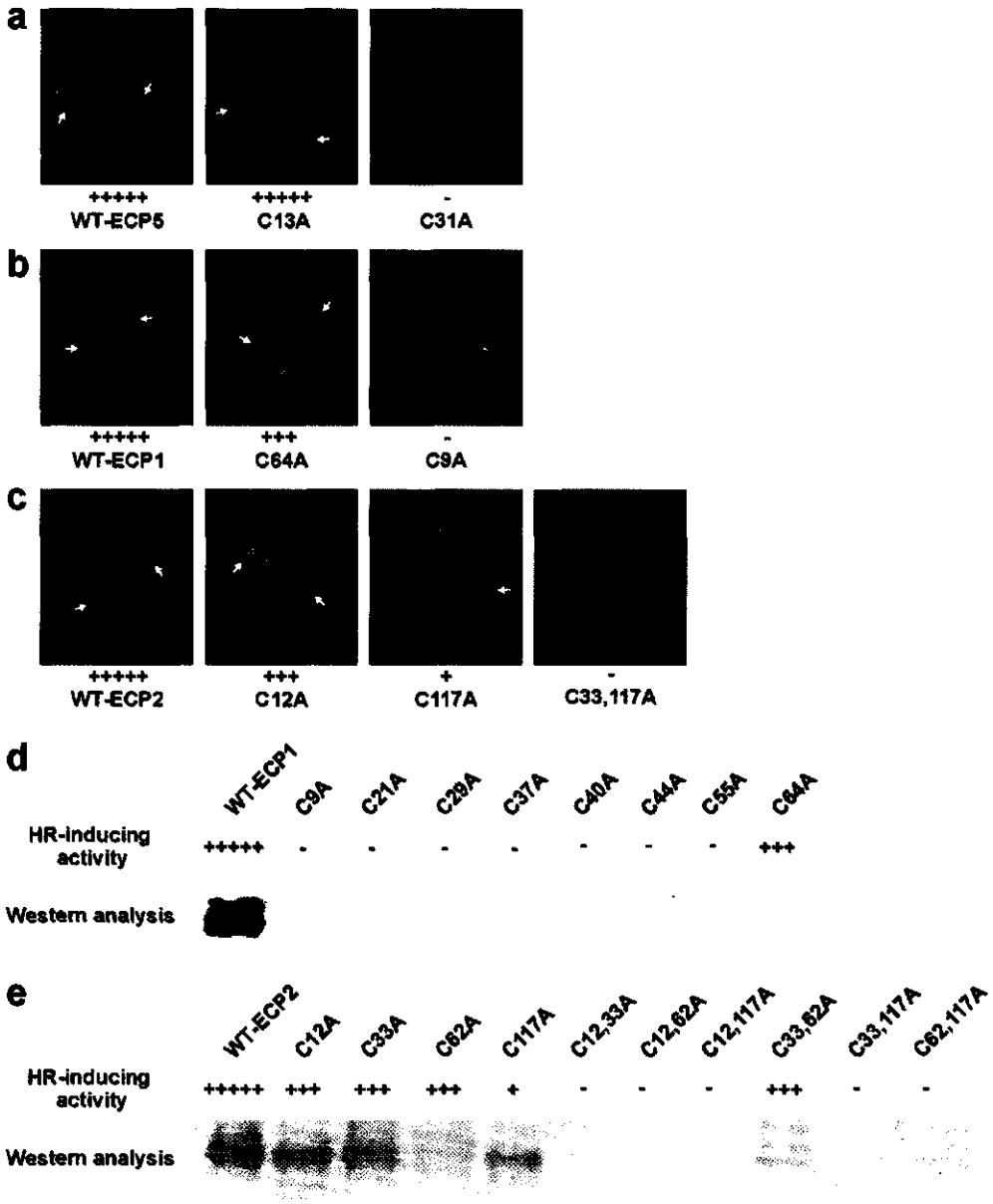

Fig. 2. Hypersensitive response (HR)-inducing activity and abundance in the apoplastic fluid (AF) of wild-type ECPs and derived mutants. Mutants are named after the position of the cysteine residue in the mature ECP protein that has been replaced by alanine. Arrows indicate epinastic leaves that show necrosis. (A) HR, visible as necrosis and epinastic symptoms, induced by PVX expressing wild-type (WT) *Ecp5* (+++++), *Ecp5* mutant C13A (+++++) and *Ecp5* mutant C31A (-), 15 days post inoculation. (B) HR induced by PVX expressing WT *Ecp1* (+++++), *Ecp1* mutant C64A (+++) and *Ecp1* mutant C9A (-), 10 days post inoculation. (C) HR induced by PVX expressing WT *Ecp2* (+++++), *Ecp2* mutant C12A (+++), *Ecp2* mutant C117A (+) and *Ecp2* double mutant C33,117A (-), 15 days post inoculation. (D) HR-inducing activity and abundance in the AF of ECP1 and derived mutants.

Fig.2 (Continued) (E) HR-inducing activity and abundance in the AF of ECP2 and derived mutants. Cysteine to alanine codon changes were introduced with a minimal amount of nucleotide changes. Mutagenic primers of 21-26 bp were designed and to introduce each mutation three PCR reactions were performed. The PVX plasmid vectors containing the wild-type *Ecp* cDNA (Laugé *et al.*, 1998 and 2000) were used as primary template. In the first PCR reaction the forward OX10-primer (5'-CAATCACAGTGTTGGCTTGC-3', annealing 5' of the *Ecp* insert in the PVX vector) and a reverse mutagenic primer were used. The second PCR reaction was performed using the reverse N31 primer (5'-GACCCTATGGGCTGTGTTG-3', annealing 3' of the *Ecp* insert in the PVX vector) and a forward mutagenic primer complementary to the primer used in the first PCR reaction. The amplified fragments were purified, combined and subjected to overlap extension PCR using the OX10 and N31 primers. The resulting fragment was digested with *Cla*I and inserted into *Cla*I-digested, dephosphorylated PVX plasmid vector (Chapman *et al.*, 1992), 3' of the duplicated coat protein promoter. PCRs were performed using *Pfu* DNA polymerase (Stratagene, Amsterdam, NL) and the sequence of the recombinant PVX constructs was verified. *In vitro* transcription and plant inoculation were performed according to Kooman-Gersmann *et al.* (1997). For assessment of the HR-inducing activity of ECP5 and derivatives, selfings of *Lycopersicon esculentum* accession G1.1161 (CfECP5, Haanstra *et al.*, 2000) were inoculated. For ECP1 and derivatives, selfings of *L. pimpinellifolium* line *Lp*-Cf-ECP1 (Laugé *et al.*, 2000) were inoculated, whereas selfings of the 'Ontario' breeding line 7518 (Cf-ECP2, Laugé *et al.*, 2000; Laugé *et al.*, 1998) were inoculated for the analysis of ECP2 and derivatives. Two to three weeks after inoculation, apoplastic fluid (AF) was isolated (De Wit and Spikman, 1982) from the third and fourth PVX-infected leaves of MM-Cf4 plants, that do not respond to the various ECPs and their derivatives. For analysis by PAGE, 75 µl aliquots of AF were freeze-dried and loaded on gel. For ECP1 detection, AF was subjected to SDS-PAGE analysis and transferred to nitro-cellulose by electroblotting. Blots were incubated with ECP1 antibodies raised against a synthetic peptide consisting of the 26 C-terminal amino acids from ECP1 (Sigma-Genosys, Cambridge, UK), at a 1:1000 dilution. For ECP2 detection, PAGE analysis at high pH in the absence of SDS was employed. Blots were incubated with polyclonal ECP2 antibodies (Wubben *et al.*, 1994) at a 1:1000 dilution. Antigen-antibody complexes were visualised using alkaline phosphatase-conjugated goat anti-rabbit IgG, under standard conditions.

This study has shown that not all cysteine residues of the ECPs are essential for full HR-inducing activity. Furthermore, there is not always a strict correlation between the amount of an ECP derivative that is present in the apoplastic fluid, and its HR-inducing activity. Beside the possible loss of a disulfide bridge, substitution of a cysteine residue by an alanine residue might affect folding (and thereby HR-inducing activity and stability) of the proteins due to intrinsic differences between these amino acids. The differences between a cysteine and an alanine residue, however, are relatively small compared to other amino acid residues. It is therefore unlikely that the observed changes in HR-inducing activity and stability of mutant ECP proteins are the result of other effects than the loss of a disulfide bridge. The loss of a disulfide bridge, however, might result in conformational changes of an elicitor protein that affect its secretion in the apoplastic space.

The data presented in this manuscript show that the role of the even amount of cysteine residues present in the ECP elicitor proteins of *C. fulvum* is more complex than anticipated. This might be the same for NIP1, a small, secreted, cysteine-rich protein of the barley pathogen *Rhynochosporium secalis*, which was proven to be both a virulence and an avirulence factor (Rohe *et al.*, 1995). Some of the cysteine residues do not seem to be involved in intramolecular disulfide bridges in the mature ECPs. They might be involved in intramolecular disulfide bridges that occur in intermediate structures during folding of the

protein, as was shown to occur during folding of AVR9 (Van den Hooven *et al.*, 1999) or they might be involved in intermolecular disulfide bridges. Such bridges could either result in homodimers or in complexes with other proteins. Currently, ECP proteins are being produced in heterologous expression systems. The production of these proteins in large amounts will allow further biochemical studies to unequivocally determine which cysteine residues of the ECP proteins are connected by a disulfide bridge. The potential formation of intermolecular disulfide bridges is of special interest, as these disulfide bridges might be important for perception of elicitor proteins by the plant as well as for their function in pathogenicity of the fungus.

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We thank H. Smid and M. Berns (Unifarm, Wageningen, The Netherlands) for excellent plant care. W. van Hof (Beeldgroep, Wageningen, The Netherlands) is acknowledged for photographing the plants. R. Luderer was supported by a grant from the Dutch Foundation for Earth and Life Sciences (ALW), project number 805-18.231.

Chapter 3

No evidence for binding between resistance gene product Cf-9 of tomato and avirulence gene product AVR9 of *Cladosporium fulvum*

This chapter has been published with minor modifications by Rianne Luderer, Susana Rivas, Thorsten Nürnberger, Benedetta Mattei, Henno W. Van den Hooven, Renier A.L. Van der Hoorn, Tina Romeis, Josa-M. Wehrfritz, Beatrix Blume, Dirk Nennstiel, Douwe Zuidema, Jacques Vervoort, Giulia De Lorenzo, Jonathan D.G. Jones, Pierre J.G.M. De Wit and Matthieu H.A.J. Joosten in Molecular Plant-Microbe Interactions 14 (7), 867-876 (2001).

Abstract

The gene-for-gene model postulates that for every gene determining resistance in the host plant, there is a corresponding gene conditioning avirulence in the pathogen. On the basis of this relationship, products of resistance (*R*) genes and matching avirulence (*Avr*) genes are predicted to interact. Here, we report on binding studies between the *R* gene product Cf-9 of tomato and the *Avr* gene product AVR9 of the pathogenic fungus *Cladosporium fulvum*. Because a high affinity binding site (HABS) for AVR9 is present in tomato lines, with or without the *Cf-9* resistance gene, as well as in other solanaceous plants, the Cf-9 protein was produced in COS and insect cells in order to perform binding studies in the absence of the HABS. Binding studies with radiolabeled AVR9 were performed with Cf-9-producing COS and insect cells and with membrane preparations of such cells. Furthermore, the *Cf-9* gene was introduced in tobacco, which is known to be able to produce a functional Cf-9 protein. Binding of AVR9 to Cf-9 protein produced in tobacco was studied employing surface plasmon resonance and surface-enhanced laser desorption and ionisation. Specific binding between Cf-9 and AVR9 was not detected with any of the procedures. The implications of this observation are discussed.

Introduction

Host specificity in plant-pathogen interactions has been described by the gene-for-gene model (Flor, 1942), which postulates that for every gene determining resistance in the host, there is a corresponding gene conditioning avirulence in the pathogen. On the basis of this relationship, products of resistance (*R*) genes and matching avirulence (*Avr*) genes are predicted to interact, resulting in an incompatible interaction (Keen, 1990). To date, a variety of *R* and *Avr* genes have been cloned (Jones and Jones, 1996; Laugé and De Wit, 1998a; Van der Biezen and Jones, 1998).

Until now, only for two R proteins, Pto from tomato and Pi-ta from rice, physical interaction with a pathogen-derived ligand, AvrPto and AVR-Pita, respectively, has been reported (Scofield *et al.*, 1996; Tang *et al.*, 1996; Jia *et al.*, 2000). Pto is a protein kinase, of which the kinase activity is required for activation of a resistance response (Sessa *et al.*, 1998). The *Pi-ta* resistance gene encodes a predicted cytoplasmic protein containing a nucleotide binding site and a leucine rich carboxyl terminus (Jia *et al.*, 2000). The number of *R* genes for which the matching *Avr* gene has been cloned is increasing (Laugé and De Wit, 1998a). This allows for more extensive studies of the mechanisms by which plant *R* gene products and corresponding pathogen-derived avirulence gene products interact and subsequently determine the outcome of the plant-pathogen interaction.

The interaction between tomato and the strictly apoplastic, biotrophic pathogen *Cladosporium fulvum* complies with the gene-for-gene model. From tomato, several *Cf* genes mediating resistance to specific races of *C. fulvum* have been cloned (Joosten and De Wit, 1999). All *Cf* genes are predicted to encode extracytoplasmic, membrane-anchored

glycoproteins that contain many leucine-rich repeats (LRRs) (Fig. 1). LRRs are thought to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). The N-terminal domains, which are highly variable, are thought to play a role in recognitional specificity, whereas the C-terminal parts of the LRRs of Cf proteins show a remarkable homology (Dixon *et al.*, 1996). For *Cf-4* and *Cf-9*, the avirulence genes encoding the corresponding race-specific elicitor of *C. fulvum*, *Avr4* and *Avr9*, respectively, have been cloned (Joosten *et al.*, 1994; Van Kan *et al.*, 1991; Van den Ackerveken *et al.*, 1992). Recognition of these elicitors by tomato genotypes carrying the matching resistance gene results in a typical hypersensitive response (HR) (Joosten *et al.*, 1994; Van den Ackerveken *et al.*, 1992).

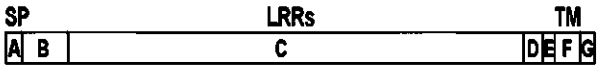

Fig. 1. Schematic representation of the Cf-9 protein. The Cf-9 protein can be divided into seven domains: A, the predicted signal peptide (SP); B, the predicted amino terminal domain of the mature protein; C, the leucine rich repeats domain (LRRs); D, a connecting domain; E, an acidic domain; F, the transmembrane domain (TM); G, a basic domain. Domains E-G anchor and orient Cf-9 in the plasma membrane in such a way that domains B-E are extracytoplasmic and domain G forms a cytoplasmic tail (Jones and Jones, 1996; Piedras *et al.*, 2000).

Upon colonisation of the intercellular spaces of tomato leaves, *C. fulvum* secretes elicitors into the apoplast (Joosten and De Wit, 1999). The apoplastic localisation of the various elicitors, together with the localisation of the LRR region of the Cf proteins on the outer surface of the plasma-membrane of the host cells (Piedras *et al.*, 2000), is consistent with the hypothesis that a direct interaction occurs between *C. fulvum*-derived elicitors and the matching Cf proteins. Although the C-terminal dilysine motif of the Cf-9 protein suggests that Cf-9 is localised in the endoplasmic reticulum (ER) (Benghezal *et al.*, 2000), this motif is not essential for Cf-9 function (Van der Hoorn *et al.*, 2001b), suggesting that functional Cf-9 protein resides in the plasma membrane, as was shown by Piedras *et al.* (2000).

The availability of near isogenic lines (NILs) of tomato containing particular *Cf* genes and of matching elicitor proteins allows for detailed studies on mechanisms of elicitor perception by a resistant host. Binding studies with radiolabeled AVR9 elicitor protein showed the presence of a high-affinity binding site (HABS) for this elicitor in plasma membranes isolated from leaves of NILs of tomato, either with or without the *Cf-9* resistance gene (Kooman-Gersmann *et al.*, 1996). Furthermore, other solanaceous plants were found to contain such a binding site, indicating that the Cf-9 protein itself is not the HABS (Kooman-Gersmann *et al.*, 1996).

Direct interaction between AVR9 and Cf-9 could not be detected employing the yeast two-hybrid system (Laurent and Honée, unpublished data). This system, however, is not entirely suitable to study interactions between such proteins. In order to perform binding studies with membrane-localised Cf-9 protein in the absence of the HABS, the Cf-9 protein was produced in COS and insect cells. Furthermore, the *Cf-9* gene was introduced in tobacco, which is known to produce functional Cf-9 protein because the injection of AVR9 in *Cf-9*-transgenic tobacco results in HR, which is visible as necrosis (Hammond-Kosack *et*

al., 1998). Here, we report on binding studies between AVR9 and Cf-9 produced in these three different expression systems. Binding studies with ^{125}I-AVR9 and Cf-9 produced by COS or insect cells were performed following the methods described by Kooman-Gersmann *et al.* (1996). Binding of AVR9 to Cf-9 protein produced in tobacco was studied employing Surface Plasmon Resonance (SPR), with BIAcore technology (Fivash *et al.*, 1998) and Surface-Enhanced Laser Desorption and Ionisation (SELDI) (Hutchens and Yip, 1993). Specific binding between Cf-9 and AVR9 was not detected with any of the procedures. The implications of this finding are discussed.

Results

Production of Cf-9 protein in COS cells.

COS cells were co-transfected with *Cf-9* fused to a mammalian signal peptide sequence and the gene encoding green fluorescent protein (GFP) (Sheen *et al.*, 1995). *GFP* expression was used to monitor the efficiency of transfection. Only those cell lines in which significant expression of *GFP* was observed were used in AVR9-binding assays. Immunolocalisation of Cf-9 revealed that the protein is predominantly present in the plasma membrane of COS cells (Fig. 2), whereas GFP accumulated in the cytoplasm and nucleus (not shown). The observation that the Cf-9 protein is present at the expected location indicates that the mammalian signal peptide directs the protein to the proper processing route, allowing posttranslational modifications. No cross-reactivity of Cf-9 antibodies was observed in untransfected COS cells (results not shown).

Binding studies with Cf-9 protein from COS cells.

Ligand binding assays with ^{125}I-AVR9 were performed with intact transgenic, Cf-9-producing COS cells and microsomal membrane preparations from such cells. Nonspecific binding was determined in the presence of an excess of unlabeled AVR9. No significant difference between total and nonspecific binding of ^{125}I-AVR9 to Cf-9-producing COS cells or to derived microsomal membrane preparations was observed (Fig. 3). Similar results were obtained with nontransgenic COS cells (results not shown). Thus, no specific binding could be detected under the conditions applied. Similar binding studies with the human Melanocyte Stimulating Hormone (MSH) receptor and ^{125}I-labeled α-MSH showed that such binding studies employing COS cells are adequate for detecting receptor-ligand interactions, with binding affinities that are similar to the affinity expected for the binding of AVR9 to Cf-9 (data not shown). Specific binding is not likely to be masked by the amount of nonspecific binding because nonspecific binding only reflects approximately 7% of the total radioligand used for binding studies with membrane preparations.

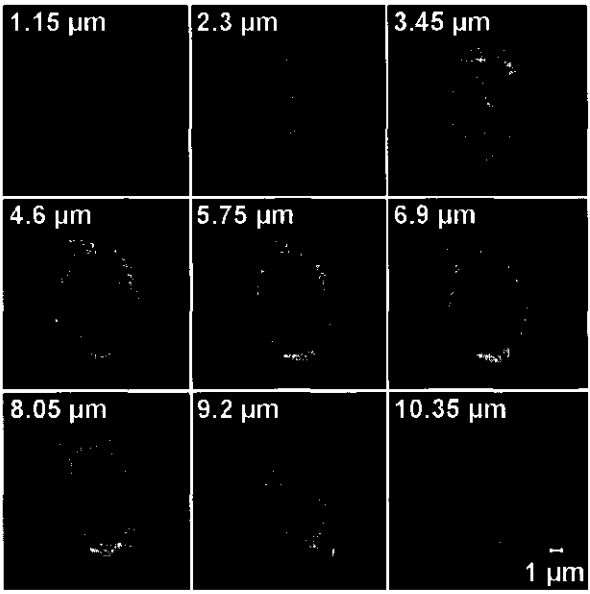

Fig. 2. Immunocytochemical visualisation of Cf-9 in COS cells. COS cells were fixed 3 days after transfection with a *Cf-9*-containing vector. Cf-9 protein was visualised with Cf-9 antibodies, combined with a secondary antibody coupled to the fluorescent dye Alexa 546. Consecutive sections of the cells are shown. The distances between dissection levels are indicated in micrometers.

In an attempt to identify appropriate conditions for radioligand binding, the experimental conditions of the AVR9 binding assay were changed sequentially. ^{125}I-AVR9 binding was not affected by alteration of the incubation temperature from 37°C to room temperature or 0°C (results not shown). Similarly, variation of the pH of the ligand binding buffer (pH 4.5, 5.5, 6.5 or 7.5) did not result in detectable specific AVR9 binding. The addition of NaCl, KCl, or KI (at 100 mM each) did not reduce nonspecific binding. Also, solubilisation of microsomal membranes, either by 1% Triton X-100 or 1% octylglucoside, which is frequently used to solubilise plasma membrane receptors, did not result in specific binding of ^{125}I-AVR9 (results not shown). Thus, none of the various conditions applied resulted in detectable, specific binding of ^{125}I-AVR9 to the Cf-9 protein produced by COS cells.

Production of Cf-9 protein in insect cells.

In addition to expression in COS cells, *Cf-9* was expressed with the baculovirus expression system. Two different recombinant baculovirus constructs were employed for production of Cf-9 protein. One recombinant virus contained the complete *Cf-9* cDNA (*Cf-9*), including the native signal sequence, whereas the other encoded the predicted extracellular part of Cf-9 (domain A to halfway through domain E, Fig. 1) containing a C-terminal His_6-tag (*Cf-9^H*).

Production of Cf-9 by baculovirus-infected insect cells was confirmed by immunoblot analysis. Different forms of Cf-9 protein are present in insect cells. Predominant forms range from approximately 84 to 125 kD for complete Cf-9 (Fig. 4). Compared with Cf-9^H, the lower mobility of the proteins produced by insect cells infected by baculovirus containing *Cf-9* is consistent with the size difference of the corresponding open reading frames. The largest form of Cf-9^H protein detected in cells infected by baculovirus

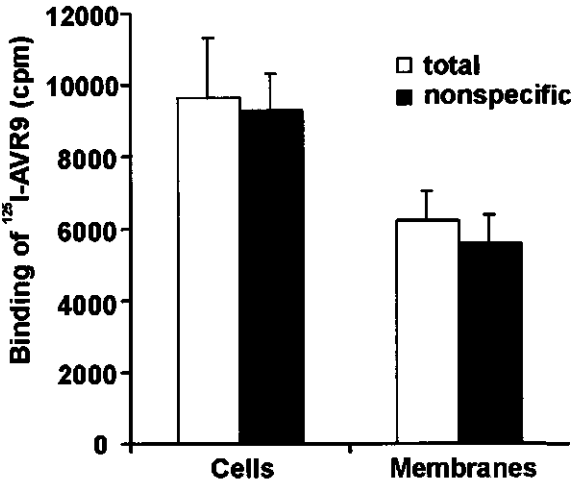

Fig. 3. Binding of ^{125}I-AVR9 to *Cf-9*-expressing COS-cells and microsomal membranes of these cells. Total binding (white columns) and nonspecific binding (black columns) are indicated in counts per minute (cpm) for cells (n=12) and microsomal membranes (n=3).

expressing *Cf-9^H* is approximately 116 kD in molecular weight. This form also was detected in culture medium, whereas no Cf-9 was detected in culture medium of cells infected by baculovirus expressing the complete *Cf-9* cDNA (Fig. 4, see arrow). This suggests that the native, N-terminal signal sequence for extracellular targeting of Cf-9 is recognised in insect cells and that the membrane anchor of the complete Cf-9 protein comprising domains E, F and G, prevents it from being secreted.

The amount of extracellular Cf-9^H produced, however, was too low to purify the protein from the culture medium. Therefore, a procedure was initiated to purify Cf-9 proteins from insect cell extracts. As the various forms of Cf-9 and Cf-9^H turned out to be insoluble under native conditions (results not shown), affinity purification of Cf-9^H was performed under denaturing conditions (see materials and methods). Purification of Cf-9^H under denaturing conditions yielded three forms that were approximately 75, 80 and 116 kD,

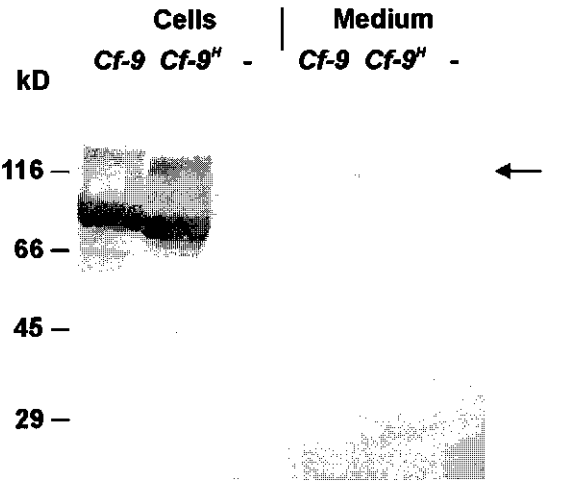

Fig. 4. Western blot analysis of Cf-9 and Cf-9^H proteins produced with the baculovirus expression system. Insect cells were infected by baculovirus containing *Cf-9* or *Cf-9^H*, or by baculovirus without an insert (-), and cultured in SF900 II medium. Proteins present in cell lysates and medium were separated by SDS-PAGE, followed by immunodetection with the use of Cf-9 antibodies. Arrow indicates the 116 kD form of Cf-9^H. Molecular weight marker positions are indicated on the left.

which all cross reacted with antibodies raised against the Cf-9 protein (Fig. 5). As these three forms were purified employing their C-terminal His_6-tag, they are all derived from a full-length translation product. The 116 kD form of Cf-9^H migrated as a more diffuse band than did the 75 and 80 kD forms. Because the Cf-9 protein contains 22 potential N-glycosylation sites (Jones *et al.*, 1994), it was anticipated that this diffuse band represents a glycosylated form of Cf-9^H. Cells that were incubated in the presence of tunicamycin, an inhibitor of N-glycosylation, did not produce the 116 kD form, whereas the 75 and 80 kD forms were still produced (results not shown), indicating that the 116 kD band indeed represents a glycosylated form of Cf-9^H. Similar results were obtained when insect cells, infected with baculovirus expressing the complete *Cf-9* cDNA, were incubated with tunicamycin. These data also indicate that approximately 41 kD of the mass of the Cf-9 protein produced in insect cells represents N-glycosylation, which contributes about 55 kD to the mass of the Cf-9 protein produced in tobacco (Piedras *et al.*, 2000). This difference is probably the result of the differences between the glycosylation in insects and plants (King and Possee, 1992). Upon processing of Cf-9^H, removal of the signal peptide occurred, because N-terminal sequencing of the 116 kD form of Cf-9^H revealed that the signal peptide had been cleaved off at the position predicted by Jones *et al.* (1994). N-terminal sequencing of the 80 and 75 kD form of Cf-9^H did not result in a clear amino acid sequence. We expect that these forms represent nonglycosylated Cf-9^H protein, either with (80 kD) or without (75 kD) signal peptide, because the estimated masses of these forms are close to the calculated masses of 88.4 and 85.9 kD respectively. These results indicate that at least part of the full-length Cf-9 protein produced in insect cells is directed to the proper processing route, allowing glycosylation and removal of the signal sequence.

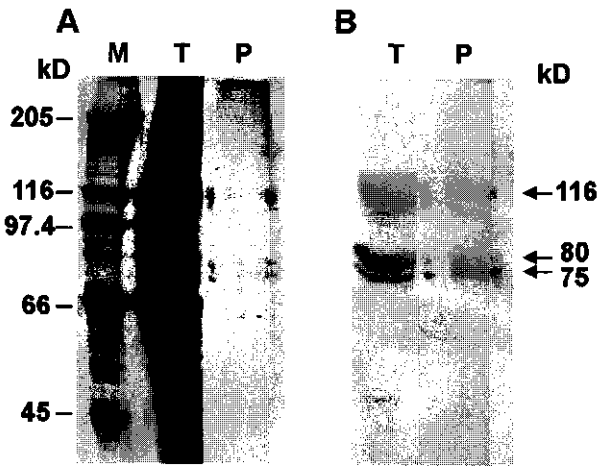

Fig. 5. Affinity purification of Cf-9^H from insect cells under denaturing conditions. Cf-9^H was purified from total insect cell proteins (T). The purified fraction (P) contains three forms of Cf-9^H of approximately 75, 80 and 116 kD (arrows). Proteins that were present in complete cell lysates and purified fractions were analysed by SDS-PAGE, followed by Coomassie staining (A) or immunodetection with the use of Cf-9 antibodies (B). Marker (M) molecular weight is indicated on the left.

Binding studies with Cf-9 proteins produced by insect cells.

Cf-9^H protein was purified under denaturing conditions. Because these conditions are expected to render the protein unsuitable for binding studies, binding assays were performed with whole insect cells producing the complete Cf-9 protein containing the membrane anchor, and with membrane-enriched fractions of such cells. Binding assays were performed in a similar way as described for binding studies with microsomal fractions of plant cells (Kooman-Gersmann *et al.*, 1996) and COS cells (this article). SDS-PAGE confirmed that the membrane-enriched fractions of Cf-9-producing insect cells also were enriched for all forms of Cf-9 (results not shown). This enrichment, however, partly is the result of the presence of insoluble Cf-9 protein that ends up in the final pellet representing the membrane-enriched fraction. Insect cells infected by baculovirus without insert and derived membrane-enriched fractions were used as a control. During all binding studies with Cf-9 produced in insect cells, microsomal fractions of MM-Cf9 tomato leaves were included as a positive control for the experimental procedures. When standard binding conditions were used, no specific binding of ^{125}I-AVR9 to Cf-9-producing insect cells or to derived membrane-enriched fractions was observed (data not shown). Binding of AVR9 to the HABS present in the microsomal fractions of tomato leaves was always detected, whereas the amount of glycosylated Cf-9 from insect cells present in binding assays is estimated to be at least 10,000-fold (membrane-enriched fractions) or 1000-fold (cells) higher than the amount of HABS present in the microsomal fractions of tomato leaves.

In an attempt to optimise binding conditions, some parameters of the binding buffer were varied. Binding studies with membrane-enriched fractions were performed at different pH values (pH 4, 5, 6, 7, 8 and 9) and salt concentrations (0, 100 and 500 mM NaCl). None of the various conditions, however, resulted in the detection of specific binding of ^{125}I-AVR9 to membrane-enriched fractions (data not shown).

As an alternative to standard binding studies, a native dot blot was made of Cf-9-producing insect cells. Mock-infected insect cells and a microsomal fraction, including the HABS, isolated from leaflets of MM-Cf9 plants, were spotted as controls,. The amount of glycosylated Cf-9 present in the spots of Cf-9 producing insect cells is estimated to be at least 1000-fold higher than the amount of HABS present in the spots of the microsomal fraction of MM-Cf9 plants. The Cf-9 protein in insect cells was readily detected by Cf-9 antibodies, whereas the amount of Cf proteins in the microsomal fraction of MM-Cf9 plants was too low to obtain a visible signal (Fig. 6, upper panel). Binding of ^{125}I-AVR9 to the spotted Cf-9-producing insect cells, however, was not observed, whereas the microsomal fraction of MM-Cf9 plants present on the blot did bind ^{125}I-AVR9 (Fig. 6, lower panel). Similar experiments were performed with blots spotted with culture medium of insect cells expressing *Cf-9^H*. Again, no specific binding of ^{125}I-AVR9 was detected (data not shown).

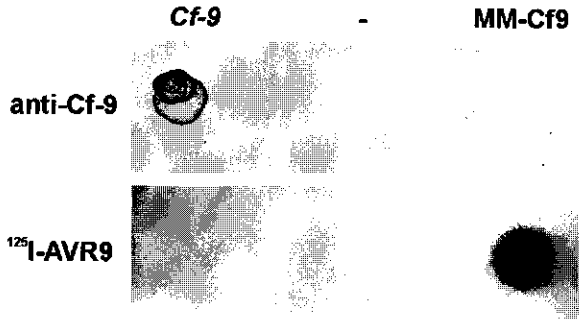

Fig. 6. Binding of ^{125}I-AVR9 to a native dot blot spotted with Cf-9-producing insect cells. Insect cells were either infected by baculovirus containing *Cf-9* (*Cf-9*) or mock infected (-) and spotted onto nitrocellulose. As a positive control, a microsomal fraction of MM-Cf9 tomato plants was spotted (MM-Cf9). Similar blots were treated with Cf-9 antibodies (upper panel) or ^{125}I-AVR9 (lower panel).

Production of Cf-9 protein in tobacco.

Because no specific binding of AVR9 to Cf-9 protein produced by COS or insect cells was observed, binding studies were performed with Cf-9 protein produced in tobacco. Transformation of tobacco with *c-myc:Cf-9* resulted in a line, designated 9161, showing HR upon injection of AVR9 (Piedras *et al.*, 2000). The c-myc:Cf-9 protein was localised in the plasma membrane (Piedras *et al.*, 2000) and could be solubilised from a microsomal fraction isolated from leaves of the 9161 line with 0.1% Nonidet P-40 (Fig. 7, lane 1). Nonidet P-40 commonly is used for the native solubilisation of membrane proteins (Hjelmeland, 1990). Nonidet P-40, however, reduces the binding of AVR9 to the HABS

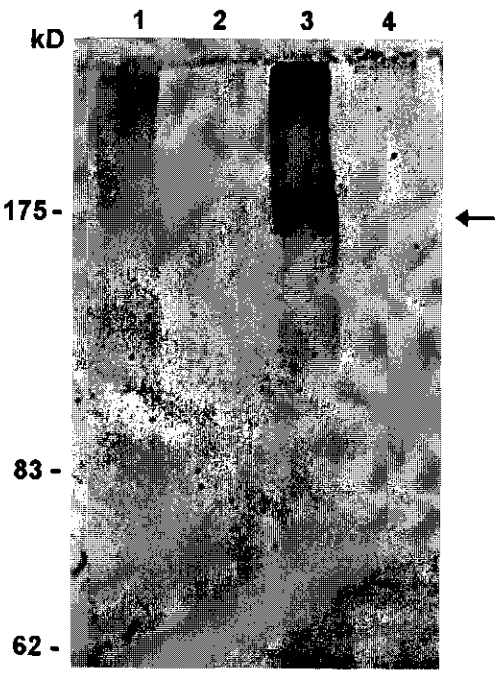

Fig. 7. Specific immunoprecipitation of c-myc:Cf-9 protein produced in tobacco by c-myc antibodies. Immunoprecipitation was carried out with microsomal preparations of leaves from *c-myc:Cf-9*-transgenic tobacco (line 9161) and c-myc monoclonal antibodies, as described in the materials and methods. The immunoprecipitated proteins were separated by SDS-PAGE and detected with the use of c-myc polyclonal antibodies. Lane 1, total microsomal fraction; lane 2, material bound nonspecifically to the protein G beads during the preclearing step; lane 3, material immunoprecipitated by the c-myc antibody; lane 4, material immunoprecipitated by unrelated hemagglutinin antibodies. Arrow indicates the position of the c-myc:Cf-9 protein. Molecular weight marker positions are indicated on the left.

detected in tobacco by Kooman-Gersmann *et al.* (1996) (Van der Hoorn, unpublished results), resulting in reduced interference of the HABS in binding studies between AVR9 and Cf-9. The solubilised c-myc:Cf-9 protein from tobacco was used in *in vitro* binding experiments, employing Surface Plasmon Resonance (SPR) and Surface-Enhanced Laser Desorption and Ionisation (SELDI) techniques.

SPR experiments with c-myc:Cf-9 protein produced in tobacco.

SPR analysis was carried out with biotinylated AVR9, which had been demonstrated to have necrosis-inducing activity (Van den Hooven *et al.*, 1999), immobilised onto the chips. Total solubilised microsomal proteins from the 9161 line or untransformed *Nicotiana tabacum* cv. Petite Havana plants (PH.4) were diluted in 20 mM sodium phosphate, pH 6.0, and injected into the flow cell. The resonance signals at the steady state (R_{eq}) that were obtained with both protein preparations were similar at comparable total solubilised microsomal protein concentrations (Fig. 8A and C). When 0.5 µg of R8K AVR9 (an AVR9 mutant showing increased necrosis-inducing activity) (Kooman-Gersmann *et al.*, 1997) was injected together with the membrane protein samples, a similar decrease in R_{eq} values was observed for proteins from the 9161 line (Fig. 8B) and PH.4 (Fig. 8D). These results indicate that the observed binding of Cf-9-containing membrane proteins to immobilised AVR9 does not reflect specific binding of Cf-9 protein, but probably represents some residual binding of the HABS in addition to nonspecific binding. Also, when a pH of 7.3 was applied, either with or without 100 mM NaCl, no specific binding of Cf-9 proteins to the

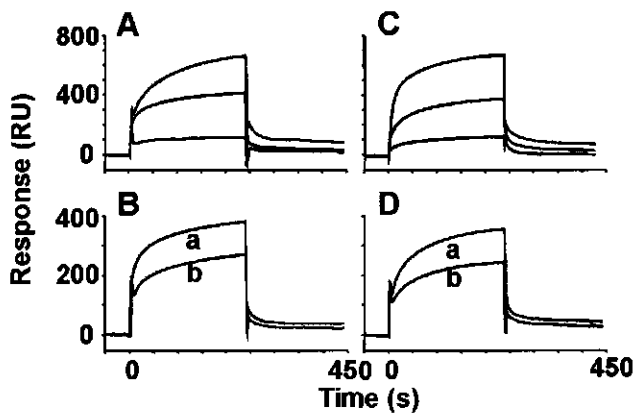

Fig. 8. Sensorgrams of the interaction between immobilised, biotinylated AVR9 and solubilised microsomal proteins from tobacco. Samples injected into the flow cell were; (A) solubilised membrane proteins from *c-myc:Cf-9*-transgenic tobacco line 9161. Increasing concentrations of membrane protein samples were used (from bottom to top curve: 30, 100 and 500 ng total proteins/µl); (B) same sample as in (A) injected at 100 ng/µl (a), and the same sample injected at 100 ng/µl, in the presence of the competing R8K mutant of AVR9 (b); (C) solubilised membrane proteins from untransformed PH.4 plants (from bottom to top curve: 30, 100 and 500 ng/µl); (D) same sample as in (C) injected at 100 ng/µl (a) and the same sample injected at 100 ng/µl, in the presence of the competing R8K mutant of AVR9 (b). Response, expressed in resonance units (RU), is reported as the difference between the signal obtained from the flow cell with immobilised AVR9 and a second flow cell without peptide. The initial baseline corresponds to the buffer flow over the AVR9 surface. An increase in RU occurs during the association phase upon sample injection. The maximum resonance signal reached during sample injection reflects the steady state, whereas the decrease in RU that occurs at the end of the injection, when sample is replaced by buffer, corresponds to the dissociation phase.

AVR9-coated chip could be detected. The observed residual binding of the HABS (Fig. 8B and D) does indicate that the experimental setup of the SPR experiments is adequate to detect binding to the immobilised AVR9. Thus, these experiments did not provide any evidence for specific binding of Cf-9 to the immobilised AVR9 peptide.

SELDI experiments with Cf-9 protein produced in tobacco.

Surface-Enhanced Laser Desorption and Ionisation (SELDI) was also used to study whether there is interaction between AVR9 and Cf-9. This technique allows determination of the mass of peptides that bind to proteins, which are covalently coupled to a chip, by measuring their time of flight with a mass spectrometer after laser irradiation.

Proteins solubilised from microsomal fractions isolated from leaves of line 9161 and the untransformed line PH.4 were immunoprecipitated with c-myc monoclonal antibodies. Cf-9 was present in the precipitate obtained from the 9161 microsomal preparation (Fig. 7, lane 3). The specificity of the antibody was confirmed by the absence of immunoprecipitate when extracts from untransformed PH.4 plants were used (not shown). No immunoprecipitated proteins were detected after the preclearing step with only protein G (Fig. 7, lane 2) and Cf-9 was not precipitated by unrelated hemagglutinin antibodies (Fig. 7, lane 4). Thus, c-myc:Cf-9 can be purified from a microsomal fraction of line 9161 by solubilisation, followed by immunoprecipitation. The results were identical when cell cultures rather than leaves of line 9161 were used to obtain microsomal fractions (data not shown).

For SELDI, immunocomplexes, obtained either from cell cultures of line 9161 or PH.4 were coupled to a protein G-coated chip. About twice as much binding of AVR9 was observed to c-myc:Cf-9-containing extracts than to extracts from PH.4 cell cultures (results not shown). Protein content and composition of the immunocomplexes originating from the cell cultures of line 9161 and PH.4, however, are probably very different. Therefore, competition experiments were carried out to further characterise the significance of the binding of AVR9 to c-myc:Cf-9-containing extracts.

Competition experiments were performed with mutants of AVR9 having higher (R8K), lower (F10A), or no necrosis-inducing activity (F21A) (Kooman-Gersmann *et al.*, 1997). The necrosis-inducing activity of these AVR9 mutants has been shown to correlate with their affinity for the HABS (Kooman-Gersmann *et al.*, 1998). The SELDI technique is sufficiently sensitive to distinguish between the molecular mass of AVR9 and the mutant peptides (results not shown). Competition experiments were performed in such a way that initially AVR9 was bound onto the immunocomplexes-treated chip and, after washing, the three different AVR9 mutants were added as competitors. There was no correlation between the ability of the AVR9 mutants to compete with AVR9 binding and their necrosis-inducing activity (Fig. 9). Similar results were obtained with the c-myc:Cf-9 protein isolated from *Cf-9*-transgenic tobacco leaves instead of from cell cultures. These results indicate that the apparent enhanced binding of AVR9 to immunoprecipitated Cf-9 is unlikely to reflect specific AVR9 binding, because it can not be competed specifically. It

has to be taken into account, however, that although there is a correlation between the necrosis-inducing activity of AVR9 mutants with their affinity for the HABS, a similar correlation does not necessarily exist with their potential affinity for Cf-9.

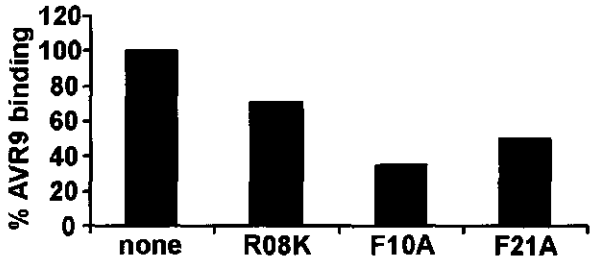

Fig. 9. Surface-enhanced laser desorption and ionisation analysis of binding between AVR9 and immobilised c-myc:Cf-9. Immunoprecipitated proteins from microsomal membranes from *c-myc:Cf-9*-transgenic tobacco line 9161 (see fig. 7) were coupled to a protein G-coated chip, which was subsequently incubated with AVR9 protein. After several washing steps (see materials and methods for details), the initial AVR9 binding value was determined. Subsequently the chip was incubated with AVR9 mutants having higher (R8K) or lower (F10A) necrosis-inducing activity than wild type AVR9 or no necrosis-inducing activity (F21A). Afterward, the AVR9 binding value was again determined. The amount of AVR9 binding detected is expressed as a percentage of the initial AVR9 binding.

Discussion

No detected binding of AVR9 to Cf-9.

We studied whether binding of race-specific elicitor AVR9 of *C. fulvum* to Cf-9, the product of resistance gene *Cf-9* of tomato, occurs. Cf-9 protein was produced in different heterologous systems, and various experimental procedures were employed to reveal whether the Cf-9 protein has binding affinity for the AVR9 elicitor. With all of the approaches we followed, however, no specific binding of AVR9 to Cf-9 was observed. The functionality of the heterologously produced Cf-9 protein and the methods used to study the potential interaction are evaluated and discussed.

Cf-9 protein was produced in COS cells, insect cells and tobacco plants as well as in cell cultures derived from these plants. The COS cell expression system and the baculovirus expression system employing insect cells, have previously been shown to be suitable for expression of various functional receptor proteins (e.g. Kieffer *et al.*, 1992; Mathews and Vale, 1991; Greenfield *et al.*, 1988; Webb *et al.*, 1989). Although there is no proof that Cf-9 produced in these systems is functional, the Cf-9 protein produced in COS cells was present in the plasma membrane (Fig. 2), whereas at least part of the Cf-9 protein produced in insect cells is directed to the proper processing route, allowing glycosylation of the protein and removal of the signal sequence. The transgenic tobacco line was shown to produce functional c-myc:Cf-9 protein, because injection of the AVR9 peptide in these plants resulted in typical necrosis (Piedras *et al.*, 2000).

Binding studies with Cf-9 produced in COS and insect cells were performed according to Kooman-Gersmann *et al.* (1996). They used ^{125}I-AVR9 and microsomal fractions or plasma membranes of leaves, to identify a HABS for AVR9 in tomato and other solanaceous plants. Considering the high amounts of heterologously produced Cf-9 protein used in the binding studies described in this article and the broad range of conditions that were tested to detect specific binding between AVR9 and Cf-9, we expected to detect specific binding if the Cf-9 protein an the AVR9 elicitor do in fact interact.

Binding of AVR9 to myc-tagged Cf-9 produced in tobacco was studied employing SPR, with BIAcore technology, and SELDI. The c-myc:Cf-9 protein was solubilised from microsomal fractions with Nonidet P-40. Although Nonidet P-40 commonly is used for the native solubilisation of membrane proteins (Hjelmeland, 1990), it cannot be excluded that Nonidet P-40 interferes with the potential binding between AVR9 and Cf-9. SPR is an excellent method to study qualitative aspects of protein-protein interactions (Fivash *et al.*, 1998) and has been shown to be a functional tool for studies on LRR proteins (Leckie *et al.*, 1999). Therefore, a comparison between microsomal protein preparations of tobacco, with or without Cf-9, could reveal binding between AVR9 and Cf-9. No difference in AVR9 binding was observed between microsomal protein fractions, however, whether they contained Cf-9 or not (Fig. 8).

SELDI ProteinChip technology (Hutchens and Yip, 1993), is a tool to study macromolecular interactions. Several researchers have reported on the successful use of SELDI to study protein-protein interactions (e.g. Davies *et al.*, 1999; Kuwata *et al.*, 1998), indicating that the use of SELDI to identify specific binding between AVR9 and Cf-9 is feasible. No correlation was found, however, between the necrosis-inducing activity of AVR9 mutants and their ability to compete with AVR9 binding (Fig. 9).

It could be argued that we failed to detect binding between AVR9 and Cf-9 because we did not identify the right experimental conditions. We consider it likely, however, that the lack of detection of specific binding of AVR9 to Cf-9, following many different approaches, reflects the absence of interaction between these proteins.

Perception of AVR9 by Cf-9.

Our results indicate that AVR9 does not directly bind to the Cf-9 protein, implying that at least a third partner is required for perception of AVR9 by Cf-9. Introduction of the *Cf-9* gene, however, has been shown to be essential and sufficient to confer the ability to respond to AVR9 to tomato, tobacco, potato and petunia (Hammond-Kosack *et al.*, 1998; Van der Hoorn *et al.*, 2000), indicating that the proposed third interacting partner is already present in these species. In the same species, a HABS for AVR9 was detected in plasma membranes (Kooman-Gersmann *et al.*, 1996). Furthermore, the necrosis-inducing activity of AVR9 mutants was shown to be correlated with their affinity for the HABS (Kooman-Gersmann *et al.*, 1998). Therefore, the HABS is a good candidate for the third interacting partner involved in the perception of AVR9 by Cf-9 (Kooman-Gersmann *et al.*, 1998; Joosten and De Wit, 1999). Whether the HABS is actually involved in the perception of

AVR9 in combination with Cf-9 has to be proven. Currently efforts are being made to purify the HABS. Hopefully, this will eventually lead to further insights into the nature of the HABS and the mechanism by which perception of AVR9 by Cf-9 takes place.

If a third interacting partner is involved in the perception of AVR9 by Cf-9, this implies that the simplest interpretation of Flor's gene-for-gene concept, direct interaction of a pathogen-derived elicitor with a matching resistance gene product (Flor, 1942; Keen, 1990), does not hold for the *Avr9/Cf-9* gene pair. Recognition mediated by a third interacting partner was suggested before for the recognition of AvrPto of *Pseudomonas syringae* by tomato (Van der Biezen and Jones, 1998), AvrRpt2 of *P. syringae* by Arabidopsis (Leister and Katagiri, 2000) and turnip crinkle virus capsid protein by Arabidopsis (Ren *et al.*, 2000). Also in other host-pathogen interactions that comply with the gene-for-gene concept, perception of a pathogen-derived elicitor by the resistance gene product might depend upon a third interacting partner. Moreover, the nature of this third partner might vary among different pathosystems. Therefore, we argue that although the genetics of the gene-for-gene concept suggest a simple direct receptor-ligand interaction, the actual biochemical mechanism of elicitor perception might be more complex than anticipated.

Materials and methods

Expression of Cf-9 *in COS cells.*

Monolayer cultivation of COS-7 cells was performed as described (Nennstiel, 1998). For transfection, 2 µg of plasmid DNA (pSeqTag vector containing Cf-9, Invitrogen, Groningen, The Netherlands) were added to $1.6x10^6$ COS-7 cells. Electroporation (250V, 500 µFd) was performed as described (Nennstiel, 1998). Electroporated cells were transferred to Petri dishes containing DMEM/FCS (Gibco-BRL, Karlsruhe, Germany). After 3 days, transfected cells were collected for preparation of microsomal membranes (see below) and AVR9 binding assays. Cotransfection with *GFP* (2 µg pCDM8/GFP) (Sheen *et al.*, 1995) was performed to monitor transfection of COS-7 cells.

For monitoring production of Cf-9 protein in COS-7 cells, cells were fixed in 4% paraformaldehyde and after blocking unspecific binding sites (10% goat serum, 3% BSA) cells were overlayed with a 1:2,500 dilution of Cf-9 antibodies. These polyclonal antibodies were raised in rabbit against the extracellular domain of Cf-9 that had been produced in *E. coli*. After incubating cell layers with a 1:2,500 dilution of the fluorescent dye Alexa 546 coupled to goat anti-rabbit IgG, localisation of Cf-9 was visualised by confocal laser microscopy.

Preparation of microsomal membranes from COS cells.

Transfected COS-7 cells were sonicated and cell debris was removed by low-speed centrifugation (1,000 x *g*). The supernatant was subjected to ultracentrifugation (100,000 x *g*) and the resulting pellet was resuspended in binding buffer (10 mM K-phosphate, pH 6.0, 0.1% BSA) and used in ligand binding assays without further treatment. Detergent solubilisation of microsomal membranes from *Cf-9*-transgenic COS-7 cells was carried out as described (Nennstiel, 1998).

AVR9 binding assays with Cf-9 producing COS cells.

Ligand binding assays were performed according to Kooman-Gersmann *et al.* (1996). If not stated otherwise, transfected COS-7 cells were resuspended in binding buffer to a final concentration of $2.5x10^6$ cells/ml. Cells (100 µl) were preincubated for 20 min at 37°C and binding was initiated by addition of 10 µl ^{125}I-AVR9 (2,200 Ci/mmol) to a final concentration of 200 pM. Nonspecific binding was determined in the presence of a 1000 fold excess of unlabeled AVR9. After incubation (3 hrs at 37°C, with occasional shaking) cells were harvested by filtration on Whatman GF/F filters. Filter-bound radioactivity was determined by crystal scintillation using a Wallac 1470 Wizard γ-counter.

AVR9 binding assays with microsomal membranes prepared from transfected COS-7 cells were carried out essentially as described above for intact cells. Microsomal membrane proteins (100 µg) were used in standard binding assays. In AVR9 binding assays with detergent-solubilised membranes, proteins were collected on Whatman GF/F filters pre-treated with 0.5% polyethylene-imine for 1 hr.

Construction of recombinant baculovirus containing Cf-9.

The complete *Cf-9* cDNA was cloned in pFastBac1 (Gibco-BRL, Breda, NL), generating pFB1-Cf-9. Furthermore, pFB1-Cf-9^H, coding for Cf-9^H protein, which lacks the predicted membrane anchor (Fig. 1) and contains a C-terminal His_6-tag, was constructed. The generated plasmids were used to obtain recombinant baculovirus via the Bac-to-Bac Expression System (Gibco-BRL, Breda, NL), according to the manufacturer's protocol.

Insect cells and baculovirus infection.

Sf21 insect cells were cultured as described (King and Possee, 1992) in Grace's medium (Gibco-BRL, Breda, NL), supplemented with 10% fetal bovine serum (Gibco-BRL, Breda, NL) in monolayers at 27°C. For Cf-9 protein production, virus inoculum was added to the cells with a multiplicity of infection of 10 plaque-forming units/cell. After 1 hr at 27°C, the inoculum was replaced by Grace's medium, supplemented with 10% fetal bovine serum or with SF900 II serum-free medium (Gibco-BRL, Breda, NL) to allow immunological analysis of the culture medium for the presence of Cf-9^H protein. To study N-glycosylation, tunicamycin (Kelly and Lescott, 1983) was added to a final concentration of 10 µg/ml, immediately after replacement of the inoculum by culture medium. Cells were harvested 72 hrs after infection and washed in phosphate buffered saline (PBS) prior to further manipulations.

SDS-PAGE and western blot analysis of Cf-9 produced by insect cells.

Insect cells were resuspended in approximately 100 µl SDS loading buffer/10^6 cells. Culture medium was freeze-dried and resuspended in SDS loading buffer to 1/10 of the original volume. After heating for 5 min at 100°C, proteins present in 10 µl of the various samples in SDS loading buffer were separated on 7.5% SDS-polyacrylamide gels and visualised with Coomassie Brilliant Blue or transferred to nitrocellulose membrane by electroblotting. The blots were incubated with the Cf-9 antibodies, which also were employed to detect Cf-9 protein in COS cells, at a 1:5,000 dilution. Antigen-antibody complexes were visualised whith alkaline phosphatase-conjugated goat anti-rabbit IgG under standard conditions. For N-terminal sequencing, purified protein was separated by SDS-PAGE and blotted onto PVDF according to Bauw *et al.* (1987). After Amido Black staining, the band of interest was excised and sequence analysis of the protein was performed by Sequentiecentrum Utrecht (Utrecht, The Netherlands).

Protein purification from insect cells.

To obtain pure Cf-9^H protein, cells were resuspended in guanidinium lysis buffer and loaded on a Xpress system His_6-affinity column (Invitrogen, Groningen, The Netherlands). The column was washed and eluted under denaturing conditions in the presence of 8M urea according to the protocol provided by the manufacturer.

Preparation of membrane-enriched fractions from insect cells.

Membrane-enriched fractions from insect cells were prepared based on the procedure of Tate and Blakely (1994) with the following modifications. The procedure was started by suspending the virus-infected insect cells in 1:20 diluted PBB (120 mM NaCl, 5 mM KCl, 50 mM Hepes-NaOH, pH 7.4). The suspension was homogenised by sonication for 20 min at 4°C. All steps were performed in the presence of 1 mM of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF). After centrifugation (12,000 x *g*), the final pellet was resuspended in MB3 buffer (250 mM sucrose, 10mM Tris HCl, pH 7.5) to a protein concentration of 1 to 10 mg/ml and stored at –80°C.

Binding studies and native dot blots with Cf-9 producing insect cells.

Binding studies were performed according to Kooman-Gersmann *et al.* (1996). For each assay, 15 to 30 µg of protein present in membrane-enriched fractions or in complete cells was used. The amount of glycosylated Cf-9 protein present in these fractions was estimated from a Coomassie Brilliant Blue-stained gel. Binding studies were performed in binding buffer (25 mM phosphate buffer, pH 6.0, 250 mM sucrose), containing 100 pM ^{125}I-AVR9, either at 37°C or room temperature (RT). Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled AVR9. In the assays performed at RT, binding conditions were varied in pH values (pH 4, 5, 6, 7, 8 or 9) and salt concentrations (0, 100 or 500 mM NaCl). GF6 glass fiber filters (Schleicher and Schuell, Einbeck, Germany) were

preincubated with polyethylamine and washed with the corresponding binding buffer before application of the samples.

For dot-blot experiments, a suspension was made of $2x10^6$ insect cells/ml of binding buffer. To disrupt the cells, the suspension was transferred twice from liquid nitrogen to a 42°C water bath and sonicated for 2 min at 4°C. Of the resulting homogenate, 10 µl was spotted onto nitrocellulose. As a positive control, 31 µg of protein of a microsomal fraction of leaves from *Lycopersicon esculentum* cv. Moneymaker tomato plants harboring *Cf-9* (MM-Cf9) (Kooman-Gersmann *et al.*, 1996) was spotted. Blots were blocked in binding buffer containing 1% BSA. After blocking, blots were incubated for 2 hrs at 37°C in 5 ml of binding buffer containing 1% BSA and 100 pM ^{125}I-AVR9. Nonspecific binding was determined in the presence of a 1,000-fold excess of unlabeled AVR9. Blots were subsequently washed for 2 hrs at 37°C in 5 ml of binding buffer and X-Omat AR film (Kodak) was exposed to the blot at –80°C in the presence of an intensifying screen.

Production of Cf-9 by tobacco and preparation of membrane protein extracts.

The transgenic tobacco line 9161 and corresponding suspension cultures, producing Cf-9 with a triple c-myc-tag in the G-domain (c-myc:Cf-9) were generated as described previously (Piedras *et al.*, 2000). The recipient plant, *Nicotiana tabacum* cv Petite Havana and the 9161 line were grown in Levington's M3 compost in the greenhouse. Suspension cultures of these lines were subcultured at 2-week intervals in Murashige and Skoog medium (Murashige and Skoog, 1962), pH 5.7, supplemented with 3% sucrose, B5 vitamins, 2,4-dichlorophenoxyacetic acid (1mg/ml) and kinetin (0.1 mg/ml).

Leaf or cell samples were ground in liquid nitrogen, thawed in 2 volumes of extraction buffer (50 mM sodium phosphate buffer, pH 7.5, 50 mM NaCl, 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride [AEBSF; Pefablock]), filtered through two layers of Miracloth and centrifuged at 1,000 x *g* for 10 min at 4°C. The supernatant was subsequently ultracentrifuged at 100,000 x *g* for 1 hour at 4°C and the microsomal membranes in the pellet were solubilised in solubilisation buffer (extraction buffer supplemented with 0.1% (octylphenoxy)-polyethoxyethanol [Nonidet P-40]) and ultracentrifuged at 100,000 x *g* for 1 hour at 4°C. The supernatant was aliquotted and stored at –70°C. The protein concentration was determined with the BCA protein assay kit (Pierce, Chester, UK), with BSA as a standard.

Surface Plasmon Resonance experiments with Cf-9 produced in tobacco.

The BIAcore X system for performing Surface Plasmon Resonance (SPR) experiments was purchased from Pharmacia Biosensor (Uppsala, Sweden). Ten microliters of 50 nM biotinylated AVR9 (Van den Hooven *et al.*, 1999) was immobilised onto streptavidin-coated sensor chips SA (Biosense Srl, Milano, Italy) at a flow rate of 5 µl/min at 25°C. Increase in the signal after immobilisation was 500 resonance units (RU). Total microsomal proteins (see above) from either *Nicotiana tabacum* cv Petite Havana or the 9161 line expressing *c-myc:Cf-9*, were diluted either in 20 mM sodium phosphate (pH 6.0) or 20 mM

HEPES (pH 7.3) with or without 100 mM NaCl. All buffers used contained 0.005% (v/v) surfactant P20 (Biosense Srl, Milano, Italy). Of these solutions 80 µl was injected into the flow cell and passed over the peptide surface at a flow rate of 20 µl/min. Binding was monitored as a mass change in the vicinity of the sensor surface, reflecting the progress of the interaction. Response, expressed in resonance units, is reported as the difference between the signal obtained from the flow cell with immobilised AVR9 and a second flow cell without AVR9 attached. Each association-dissociation cycle was followed by a regeneration phase, during which the flow cell was flushed with 20 µl of HBS buffer (10mM Hepes, pH 7.4, 150 mM NaCl, 0.005% [v/v] surfactant P20 (Biosense Srl, Milano, Italy), in distilled water) to remove bound proteins.

Immunoprecipitation and immunoblotting of protein extracts from tobacco.

Prior to immunoprecipitation, the aliquots of either leaf- or cell-solubilised microsomes were thawed and ultracentrifuged at 100,000 x *g* for 30 min at 4°C. From the supernatant, a volume containing 250-500 µg of protein was precleared in a final volume of 500 µl of solubilisation buffer (see above), for 1 hour at 4°C in the presence of 5% (v/v) protein G coupled to beads. The mixture was centrifuged and the supernatant was incubated with c-myc monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at a final dilution of 1:50. After 3 hours at 4°C, the immunocomplexes were either used for SELDI experiments (see below) or western blotting. In the latter case, after incubation with 5% (v/v) protein G coupled to beads for 1 hour at 4°C, the precipitate was collected by centrifugation, washed three times with solubilisation buffer, and subsequently resuspended in SDS-sample buffer.

Precipitated proteins were separated on a 7.5% SDS gel and transferred onto nitrocellulose (Amersham Pharmacia Biotech, Little Chalfont, UK) by wet electroblotting with the Mini-Protean II system (Bio-Rad, Hemel Hempstead, UK). The blots were incubated with c-myc polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a final dilution of 1:2,000 for 1 hour and subsequently antigen-antibody complexes were visualised with alkaline phosphatase-conjugated goat anti-rabbit IgG under standard conditions.

SELDI experiments with Cf-9 produced in tobacco.

SELDI (Surface-Enhanced Laser Desorption and Ionisation) assays were performed with the Ciphergen SELDI™ system (Ciphergen Biosystems Ltd, Palo Alto, CA, USA). Binding experiments were carried out with preactivated surface chips. Preactivation of the chips was carried out following the manufacturer's recommendations. All incubations were performed in a humidity chamber at RT. One microliter of protein G (0.8 mg/ml in PBS buffer, pH 7.3) and 1 µl of acetonitrile were spotted on every slot. Covalent linking of protein G to the chip was carried out for 1 hour. After removing excess protein G, residual sites on each slot were blocked for 20 min with 4 µl of 1 M ethanolamine in PBS, pH 7.2. The chip was first

washed with buffer A (20 mM sodium phosphate buffer, pH 7.0, 500 mM NaCl, 0.1% Nonidet P-40) for 5 min, and then with buffer B (20 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl, 0.1% Nonidet P-40) for 5 min. Immunoprecipitated complexes formed between the c-myc monoclonal antibodies and proteins in the solubilised microsomal fractions (see above), were coupled to the protein G-coated chip for 1 hour and washed in four steps: 5 min with buffer B, 1 min with buffer A, 5 min with buffer B and 5 min with 20 mM sodium phosphate (pH 7.0). Subsequently, 1 µl of a solution of 10 µM AVR9 peptide in binding buffer (20 mM sodium phosphate, pH 6.0, 0.1% Nonidet P-40) was spotted and allowed to incubate for 1 hour. Afterward, the chip was washed three times with washing buffer (20 mM sodium phosphate, pH 6.0, 500 mM NaCl, 0.1% Nonidet P-40), once with 10 mM HEPES buffer (pH 7.0) and then dried. For competition experiments the chip was treated similarly. After incubation with AVR9 and subsequent washing steps, however, a 100-fold excess of a competitor peptide was added. After 1 hour incubation, the chip was washed as described above. Finally 0.5 µl of matrix [α-cyano-2'-hydroxycinammic acid (CHCA), (5 mg/ml in 30% acetonitril, 0.1% trifluoroacetic acid)] were added to each slot. For the estimation of AVR9-binding values, the adrenocorticotropic hormone (ACTH) was used as an internal standard. The ratio between the ACTH peak and AVR9 peak was calculated in all cases.

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Chapter 4

Cladosporium fulvum overcomes *Cf-2*-mediated resistance by producing truncated AVR2 elicitor proteins

This chapter is submitted for publication by Rianne Luderer, Frank L.W. Takken, Pierre J.G.M. de Wit and Matthieu H.A.J. Joosten

Abstract

The *Cf-2* gene of tomato confers resistance to strains of the biotrophic pathogenic fungus *Cladosporium fulvum* carrying avirulence gene *Avr2*. To allow dissection of the biochemical mechanism of perception of AVR2 by Cf-2, we set out to clone the *Avr2* gene. Here we report the functional cloning of *Avr2* cDNA, based on the induction of a hypersensitive response (HR) by the encoded AVR2 protein in Cf2 tomato plants. Analysis of strains of *C. fulvum* that are virulent on Cf2 tomato lines revealed various independent frame-shift mutations in the *Avr2* ORF and a point mutation resulting in a premature stopcodon. All modifications result in the production of truncated AVR2 proteins. Interestingly, an additional modification involves the insertion of a LINE-like element, Cfl1, in the *Avr2* ORF. Cfl1 is the first LINE-like element identified in *C. fulvum* and provides the first example of loss of avirulence of a plant pathogen due to insertion of a retrotransposable element in an *Avr* gene. Rcr3 represents an additional plant protein that is specifically required for *Cf-2*-mediated resistance. Analysis of two different rcr3-mutant Cf2 tomato plants revealed that their ability to respond to AVR2 with a HR correlates with their degree of resistance to AVR2-producing strains of *C. fulvum*. These data support a role of Rcr3 in the perception of AVR2 by Cf-2.

Introduction

Host specificity in plant-pathogen interactions has been described by the gene-for-gene model (Flor, 1942, 1946). This model postulates that for every dominant gene determining resistance in the host plant, there is a matching dominant gene conditioning avirulence in the pathogen. The simplest biochemical model for perception of an avirulence (AVR) protein by a resistant host plant involves direct interaction of the AVR protein with the matching resistance (*R*) gene product (Gabriel and Rolfe, 1990; Keen, 1990). Perception of an avirulence protein by the host plant elicits a hypersensitive response (HR), culminating into resistance. To date, a variety of *R* and *Avr* genes have been cloned (reviewed by Takken and Joosten, 2000a and Van 't Slot and Knogge, 2002). The number of host-pathogen relationships, however, for which a direct interaction between *R* and *Avr* gene products has been detected, is still very limited (reviewed by Luderer and Joosten, 2001a). In fact, for most gene-for-gene relationships studied so far, experimental evidence is more consistent with indirect perception of an AVR protein by an R protein, than with a direct physical interaction between these proteins (reviewed by Luderer and Joosten, 2001a). Indirect perception of an AVR protein by an R protein implies that at least a third component is required for specific recognition.

The interaction between tomato (*Lycopersicon spp.*) and the biotrophic fungus *Cladosporium fulvum* complies with the gene-for-gene model. The tomato *R* genes *Cf-2*, *Cf-4*, *Hcr9-4E*, *Cf-5* and *Cf-9* confer resistance to strains of *C. fulvum* that carry the corresponding *Avr* gene, *Avr2*, *Avr4*, *Avr4E*, *Avr5* and *Avr9*, respectively (reviewed by

Joosten and De Wit, 1999). All *Cf* genes are predicted to encode a receptor-like protein with an extracellular LRR region, a transmembrane domain and a short cytoplasmic tail with no homology to known signalling domains (reviewed by Joosten and De Wit, 1999).

Growth of *C. fulvum* is confined to the apoplastic space of tomato leaves (De Wit, 1977). Two race-specific avirulence proteins of *C. fulvum*, AVR9 and AVR4, were isolated from apoplastic fluid and the corresponding genes have been cloned (Scholtens-Toma and De Wit, 1988; Van Kan *et al.*, 1991; Van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). Consistent with the presence of the AVR proteins in the apoplastic space, both *Avr9* and *Avr4* encode a protein with a signal peptide for extracellular targeting (Van Kan *et al.*, 1991; Joosten *et al.*, 1994). The *Avr9* gene encodes a precursor protein of 63 amino acids (aa), that is further processed by fungal and plant proteases into a 28-aa peptide that contains six cysteine residues (Van Kan *et al.*, 1991; Van den Ackerveken *et al.*, 1993b). These cysteine residues were shown to be involved in disulfide bridges (Van den Hooven *et al.*, 2001). In strains of *C. fulvum* that are virulent on Cf9 tomato plants, the *Avr9* gene is absent (Van Kan *et al.*, 1991). *Avr4* encodes a 135-aa pre-pro-protein, which upon secretion is N- and C-terminally processed, resulting in an 86-aa mature AVR4 protein (Joosten *et al.*, 1994; Joosten *et al.*, 1997). The mature AVR4 protein contains eight cysteine residues, which are thought to be involved in disulfide bridges. Strains of *C. fulvum* that are virulent on Cf4 tomato plants show various single point mutations in the *Avr4* ORF, that result in the production of unstable AVR4 isoforms (Joosten *et al.*, 1997).

Extensive binding studies have been performed to detect whether direct interaction between avirulence protein AVR9 and resistance protein Cf-9 occurs. Although various experimental approaches were followed, no binding between AVR9 and Cf-9 was detected (Luderer *et al.*, 2001b). These results indicate that perception of AVR9 by Cf-9 is indirect, which implies that at least a third component is required. The high-affinity binding site (HABS) for AVR9 that has been identified in several solanaceous species (Kooman-Gersmann *et al.*, 1996), might be the third component that is required for perception of AVR9 by Cf-9. The binding affinity of mutant AVR9 peptides for the HABS was shown to correlate with their ability to induce a HR in tomato lines carrying *Cf-9* (Kooman-Gersmann *et al.*, 1998). Thus far, however, attempts to purify the HABS to clone its encoding gene by reversed genetics, have not been successful (Van der Hoorn, 2001a). Currently, binding studies are performed with AVR4 and Cf-4 to unravel whether a direct interaction between these proteins takes place (Westerink, unpublished data).

Recently, the *Rcr3* gene (for required for *C. fulvum* resistance 3) that is required for *Cf-2*-mediated resistance, was identified (Dixon *et al.*, 2000). *Rcr3* is specifically required for *Cf-2*-mediated resistance but not for *Cf-5*- and *Cf-9*-mediated resistance (Dixon *et al.*, 2000). The *Rcr3* gene has been isolated and the predicted gene product shows homology to secreted cysteine proteases (J. Krueger, C.M. Thomas, C. Golstein, M.S. Dixon and J.D.G. Jones, personal communication). Cf-2 and Cf-5 are thought to activate the same defence signalling pathway upon perception of the matching AVR protein, as Cf-5 is over 90% identical to Cf-2 (Dixon *et al.*, 1996; Dixon *et al.*, 1998). Rcr3 has been proposed to play a

role upstream of this common defence pathway and might represent the third component that is required for perception of AVR2 by Cf-2 (Dixon *et al.*, 2000). The predicted extracellular localisation of Rcr3 is consistent with this hypothesis. To allow dissection of the biochemical mechanism of perception of AVR2 by Cf-2, we set out to clone *Avr2*. Several attempts to purify the AVR2 protein from intercellular fluid obtained from a compatible interaction between tomato and an AVR2-producing strain of *C. fulvum* were unsuccessful (Paul Vossen and Matthieu Joosten, unpublished results). Therefore we set out to clone *Avr2* cDNA by employing a functional screen based on the HR-inducing activity of the AVR2 protein in Cf2 tomato plants (Takken *et al.*, 2000b).

Here we report the functional cloning of *Avr2* cDNA by employing a potato virus X (PVX)-based binary expression vector. By transformation of *C. fulvum* with a genomic clone of *Avr2* we show that production of AVR2 confers avirulence of the fungus on Cf2 tomato plants. Strains of *C. fulvum* that are virulent on Cf2 tomato plants contain various modifications in the *Avr2* ORF. These modifications all result in the production of truncated AVR2 proteins. Interestingly, one of the modifications involves the insertion of a LINE-like element (also known as non-long-terminal-repeat (non-LTR) retrotransposable elements). Analysis of two different rcr3-mutant Cf2 tomato plants revealed that their ability to respond to AVR2 correlates with their degree of resistance to AVR2-producing strains of *C. fulvum*. The putative role of Rcr3 in the perception of AVR2 by Cf-2 will be discussed.

Results

Functional cloning of Avr2 *cDNA*

To clone *Avr2*, a binary, PVX-based cDNA library obtained from *C. fulvum* grown *in vitro* (Takken *et al.*, 2000b) was expressed in Cf2 tomato plants. The strain of *C. fulvum* that was used to create this library (strain 5a, Takken *et al.*, 2000b) is avirulent on Cf2 tomato plants, which indicates that it carries avirulence gene *Avr2*. The 9600 *Agrobacterium tumefaciens* colonies comprising the cDNA library were toothpick-inoculated on leaflets of Cf2 tomato plants and as a negative control Cf9 tomato plants were inoculated in parallel. Upon inoculation of the plant, *A. tumefaciens* transfers the PVX expression vector to the plant cells and subsequently PVX particles are formed that spread around the inoculation site (Takken *et al.*, 2000b). When the virus expresses a *C. fulvum* cDNA that encodes the AVR2 protein, a spreading HR is expected to be specifically induced in leaflets of Cf2 tomato plants. After a prudent selection, 61 putative positive clones that induced a HR on Cf2 tomato plants were identified in the first screen. Repeated screening revealed that two of these clones reproducibly induced a HR specifically upon expression in Cf2 plants. Sequencing revealed that both clones contained an identical open reading frame (ORF) of 237 bp, which we tentatively designated *Avr2*. The clones were independent, as their 3' - ends were different. The sequence of the largest cDNA clone has been filed in the EMBL database under accession number AJ421629.

The *Avr2* ORF encodes a predicted protein of 78 aa, which contains 8 cysteine residues (Fig.1). The AVR2 protein was analysed with the SignalP program (Nielsen *et al.*, 1997), which predicted a 20-aa N-terminal signal sequence for extracellular targeting. The *Avr2* cDNA-sequence does not have significant homology with sequences present in databases or with other *Avr* genes of *C. fulvum*. Database-searches with the predicted AVR2 protein did not reveal significant homology either.

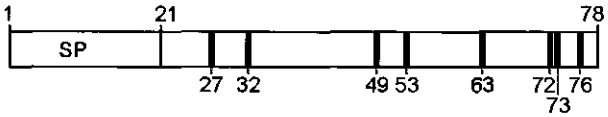


Fig. 1. Schematic representation of the AVR2 protein. The protein contains a predicted signal peptide (SP) for extracellular targeting and eight cysteine residues, indicated as black bars. Numbers indicate the amino acid positions.

Avr2 cDNA was used to screen a genomic library of *C. fulvum* (see Experimental procedures). This screen resulted in the identification of 8 independent clones ranging from about 12 kb till over 20 kb that all contained the complete *Avr2* ORF. Partial sequencing of two of these clones and comparison of the sequence with the cDNA sequence of *Avr2*, revealed that the *Avr2* gene contains one intron of 54 bp which is positioned after bp 159 of the *Avr2* ORF. The genomic sequence of *Avr2* was determined from 663 bp upstream till 652 bp downstream of the ORF. This sequence has been filed in the EMBL database under accession number AJ421628.

Expression of Avr2 *results in a* Cf-2*-specific HR*

The *Avr2* cDNA was cloned from a library derived from an *in vitro* culture of *C. fulvum*. To examine the expression of the gene during growth of the fungus *in planta*, a strain of *C. fulvum* which is avirulent on Cf2 tomato plants was inoculated onto susceptible Cf0 tomato plants, which do not harbour any known functional *Cf* resistance gene. At several days after inoculation, infected leaves were harvested for RNA isolation. Figure 2 shows that the *Avr2* gene of *C. fulvum* is expressed during growth of the fungus on tomato. The increase of the transcript level of the fungal actin gene over time indicates successful colonisation of the leaves by *C. fulvum*.

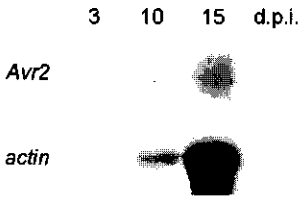
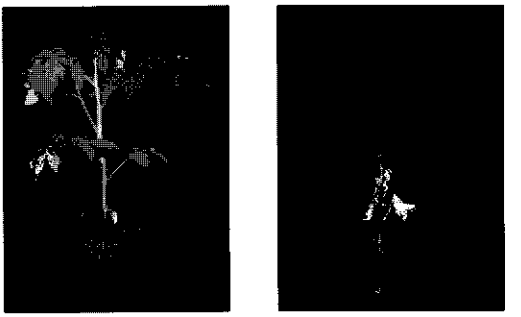


Fig. 2. Expression of *Avr2* during growth of *C. fulvum* on susceptible Cf0 tomato plants. A Northern blot of total RNA from infected tomato leaves, isolated at 3, 10 and 15 days post inoculation (d.p.i.), was probed with DNA fragments of the *Avr2* ORF or the *C. fulvum* actin gene.

To further confirm the specific HR-inducing activity of AVR2 upon its production in Cf2 tomato plants, the *Avr2* ORF was amplified from the cDNA and cloned in a PVX-based expression vector, resulting in PVX::*Avr2* (see Experimental procedures). Figure 3 shows that PVX-mediated expression of *Avr2* results in severe systemic necrosis in Cf2 tomato plants, whereas inoculation of Cf2 tomato plants with PVX without an *Avr2* insert (PVX::-), only results in typical systemic mosaic symptoms caused by the viral infection. Tomato plants carrying resistance gene *Cf-4* and *Hcr9-4E*, *Cf-5*, *Cf-9*, *Cf-ECP2* (Laugé *et al.*, 1998b) or *Cf-ECP5* (Haanstra *et al.*, 2000) that were inoculated with PVX::*Avr2* or PVX::-, only developed systemic mosaic symptoms (results not shown). Together, these data show that expression of *Avr2* in tomato induces a *Cf-2*-specific HR.

Fig. 3. Phenotype of Cf2 tomato plants inoculated with PVX::*Avr2* (right) or PVX::- (left). Plants were photographed 14 days after inoculation with PVX. Note the AVR2-induced necrosis (right).



PVX::- PVX::*Avr2*

Avr2 *is the genuine avirulence gene matching* Cf-2

To prove that *Avr2* confers avirulence of *C. fulvum* on Cf2 tomato plants, a strain of *C. fulvum* that is virulent on Cf2 tomato plants (designated #31, producing all known AVR proteins except for AVR2, see below), was co-transformed with a genomic clone of *Avr2* and a hygromycin selection marker (see Experimental procedures). Twenty-four hygromycin-resistant *C. fulvum* transformants were selected and inoculated onto Cf0 and Cf2 tomato plants. All transformants were still able to colonise Cf0 tomato plants, indicating that the transformation procedure did not affect their pathogenicity. In contrast to the recipient strain, however, 10 out of the 24 hygromycin-resistant transformants had become avirulent on Cf2 plants (see Fig. 4 for representative symptoms). Standard PCR analysis (see Experimental procedures) revealed a strict correlation between the presence of the *Avr2* transgene and the inability to colonise Cf2 tomato lines (results not shown). Transformants that were still virulent on Cf2 tomato plants only contained the hygromycin selection marker and lacked the *Avr2* transgene. These results demonstrate that *Avr2* confers avirulence of *C. fulvum* on Cf2 tomato plants, thereby rendering *Avr2* a genuine avirulence gene.

To show that the *C. fulvum Avr2*-transformants produce AVR2 protein, apoplastic fluid was isolated from Cf0 tomato plants that were colonised by either transformants or the recipient strain. All apoplastic fluids induced necrosis upon injection into leaves of Cf9

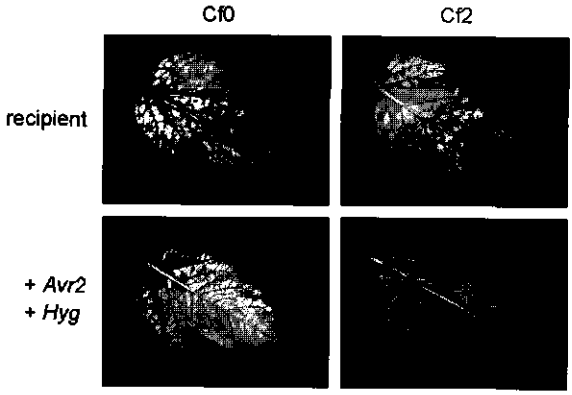


Fig. 4. *Avr2* confers avirulence of *C. fulvum* on Cf2 tomato plants. Cf0 and Cf2 tomato plants were inoculated with a strain of *C. fulvum* that is virulent on Cf2 tomato plants (recipient) and an *Avr2*-transformant of that strain (+ *Avr2* + *Hyg*). Colonisation by the fungus is visible as white mycelium on the lower side of the tomato leaf. Leaves were photographed 15 days after inoculation with the fungus. Note that no fungal colonisation takes place after inoculation of Cf-2 tomato plants with an *Avr2*-transformant.

plants (Fig. 5), indicating that the apoplastic fluids contain AVR9. The presence of AVR9 confirms that the apoplastic fluids were isolated from leaves that were successfully colonised by strain #31 or derived transformants. Only the apoplastic fluids that were isolated from leaves that were colonised by *C. fulvum* transformants that contain the *Avr2* transgene induced necrosis upon injection in leaves of Cf2 tomato plants (Fig. 5). These results show that only the *C. fulvum* transformants that contain the *Avr2* transgene and had become avirulent on C2 tomato plants, secrete AVR2 protein in the apoplastic space during colonisation of tomato leaves. Altogether, these data show that transformation of *C. fulvum* with *Avr2* results in avirulence of the fungus on Cf2 tomato plants, due to production of the AVR2 protein.

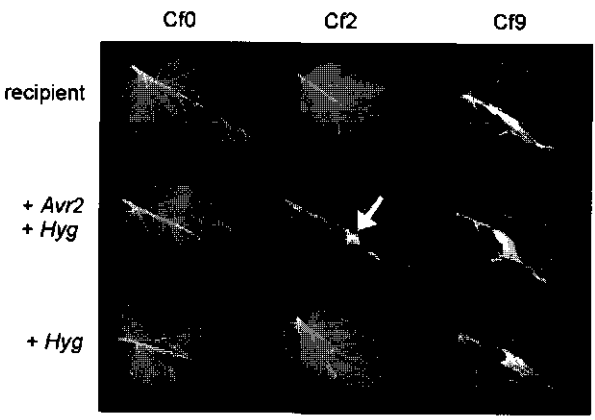


Fig. 5. *C. fulvum Avr2*-transformants secrete AVR2 in the apoplastic space. Apoplastic fluid was isolated from tomato leaves that were successfully colonised by the recipient strain, a transformant that contains the *Avr2* transgene (+ *Avr2*, + *Hyg*) or a transformant that only contains the hygromycin selection marker (+ *Hyg*). Leaves of Cf0, Cf2 and Cf9 tomato plants were injected with these apoplastic fluids and photographed at 6 days after injection. Note that in Cf2 tomato leaves only injection of apoplastic fluid isolated from tomato leaves that were colonised by the *C. fulvum Avr2*-transformant results in a HR, visible as necrosis (see arrow).

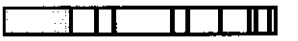
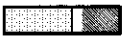
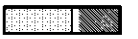
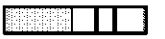
C. fulvum *circumvents* Cf-2*-mediated resistance by various modifications in* Avr2

Southern blot analysis of genomic DNA of various strains of *C. fulvum* revealed that *Avr2* is present as a single copy gene in the genome of strains that are avirulent on Cf2 tomato plants (results not shown). However, also in the genome of strains that are virulent on Cf2 tomato plants, a single fragment hybridising with *Avr2* is present (results not shown). Northern blot analysis of RNA isolated from compatible interactions involving several strains of *C. fulvum* revealed that, except for two strains (#31 and #41) for which no hybridisation was detected (see below), *Avr2*-hybridising transcripts are produced by all strains (results not shown). As strains that are virulent on Cf2 tomato plants are not expected to produce a functional AVR2 protein, the *Avr2*-homologous sequence of all *C. fulvum* strains present in our collection that are virulent on Cf2 plants, was PCR-amplified from the genome (using standard conditions, see Experimental procedures) and sequenced. For all strains that are virulent on Cf-2 tomato plants, a mutation was detected in the *Avr2* ORF (listed in Table 1). Most modifications are frameshift mutations, resulting from single nucleotide insertions or deletions, which were identified at several sites in the *Avr2* ORF. Furthermore, in four strains a point mutation, resulting in the introduction of a premature stopcodon was found. All the modifications of the *Avr2* ORF that were identified are predicted to result in a truncated AVR2 protein (see Table 1). We therefore conclude that *C. fulvum* circumvents *Cf-2*-mediated resistance by producing truncated AVR2 proteins. Compared to the wild-type AVR2 protein, the truncated AVR2 proteins lack at least three cysteine residues (see Table 1) and are therefore expected to be unstable and/or non-functional. The sequence of the *Avr2* ORF of 15 strains that are avirulent on Cf2 tomato plants was identical to the sequence that was originally identified. Furthermore, sequencing revealed that, compared to the initially identified genomic sequence of *Avr2*, some strains lack 3 nucleotides (TGA) at position 27 till 29 of the intron of *Avr2*. This deletion apparently does not influence the splicing of the intron, as it is present in strains that are virulent on Cf2 plants as well as in strains that are avirulent on Cf2 plants.

Insertion of a LINE-like element in Avr2 *results in circumvention of* Cf-2*-mediated resistance for two strains of* C. fulvum

PCR amplification of the *Avr2* gene using standard conditions did not result in a product for the two strains (#31 and #41) for which an *Avr2*-transcript was not detected by Northern analysis. The application of PCR conditions optimised for amplification of long DNA fragments (see Experimental procedures), however, resulted in a PCR product for these two strains of about 5.3 kb, which is almost 5 kb longer than the product generated from all other strains. The 5.3 kb PCR fragments were initially sequenced partially (see Experimental procedures) and both strains turned out to contain an insert, which was located after bp 55 of the *Avr2* ORF. The inserts present in both strains were identical for the parts that were sequenced (785 bp of the 5'-end and 550 bp of the 3'-end of the insert). Restriction analysis of the insert with 12 different restriction enzymes with a 6-bp

Table 1. Mutations in the *Avr2* ORF of *C. fulvum* strains that are virulent on Cf2 tomato plants.

# strains[a]	mutation	nt position in ORF	predicted protein[b]
15[c]	-		
4	C to T (stop)	198	
2	Δ T	214	
1	Δ C	70	
11	Δ A	69	
1	+ A	after 119	
7	+ A	after 69	
2	+ 5 kb[d]	after 55	

[a] Number of strains in which the mutation was identified.

[b] For each mutation the predicted effect on the AVR2 protein is indicated. The dotted area represents the signal peptide of AVR2. Cysteine residues of AVR2 are indicated as black bars. The hatched areas represent amino acids encoded after a frameshift mutation in the *Avr2* ORF.

[c] These15 strains are avirulent on Cf2 tomato plants and do not contain any mutation in the *Avr2* ORF.

[d] See figure 6 for more details.

recognition site did not reveal any polymorphisms either. After the insertion, a duplication of 15 bp of the *Avr2* ORF that were preceding the insertion site was identified. From *C. fulvum* strain #31, the 5 kb insert in the *Avr2* ORF was sequenced completely (single stranded), employing a primer-walking approach. BlastX homology searches (Altschul *et al.*, 1997) revealed that the insert sequence contains a region of which the translation product has homology with gag proteins and another region of which the translation product has homology with reverse transcriptases (see Fig. 6 for a schematic representation). The orientation of the protein-encoding regions of the insert is opposite to that of the *Avr2* ORF. However, as the insert was only sequenced single-stranded, a reliable determination of the exact ORFs is not feasible. The overall features of the insert show resemblance to non-long-terminal-repeat (non-LTR) retrotransposable elements, which are also called LINE-elements. The insert was therefore named Cfl1, for *C. fulvum* LINE-like element 1. Translation of nucleotide 1033 till 1112 of *Cfl1* reveals the presence of three $CysX_2CysX_4HisX_4Cys$ motifs, of which the second and the third motif are slightly degenerate. This is a typical motif for the first ORF of LINE-like elements (Cambareri *et al.*, 1994). The region of *Cfl1* of which the encoded protein shows homology with reverse transcriptases, shows the highest homology with a reverse transcriptase of Tad3-2 and Tad1-1, which are LINE-like elements of *Neurospora crassa* (Cambareri *et al.*, 1994). Moreover, as mentioned above, the insertion site of Cfl1 showed a 15-bp target site

duplication. Altogether, these data indicate that Cfl1 represents a novel LINE-like element of *C. fulvum*. The sequence of Cfl1 has been filed in the EMBL database under accession number AJ421630.

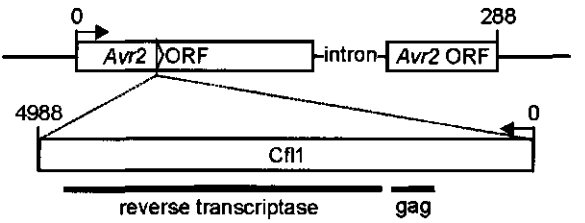


Fig. 6. Schematic representation of the *Avr2* gene and the Cfl1 insert that was identified in two strains of *C. fulvum*. The regions of Cfl1 that encode proteins with homology to gag proteins and reverse transcriptases are indicated. Arrows indicate the orientation of the ORFs. The 15-bp target site duplication is indicated with a triangle. Numbers indicate nucleotide positions. Note that the size of the Cfl1 element is not in proportion to the size of the *Avr2* ORF.

Specific recognition of the AVR2 protein by Cf2 tomato and the requirement of Rcr3

The *Cf-2* resistance locus contains two functional resistance genes, *Cf-2.1* and *Cf-2.2*, which differ only by three aa (Dixon *et al.*, 1996). Because of this feature, it was anticipated that they both confer recognition of the same avirulence protein, AVR2. Thus far, however, it could not be excluded that they each recognise a different AVR protein. For this reason, Cf0 tomato plants, transformed with either *Cf-2.1* or *Cf-2.2* (Dixon *et al.*, 1996), were inoculated with PVX::*Avr2*. Figure 7 shows that both transgenic lines developed severe necrotic symptoms, whereas the untransformed Cf0 plant only developed systemic mosaic symptoms typical for the viral infection. These data prove that indeed both *Cf-2.1* and *Cf-2.2* mediate AVR2 recognition.

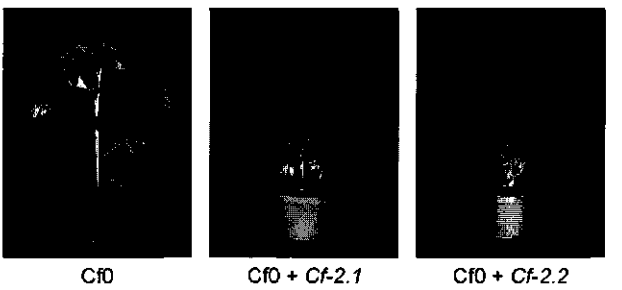


Fig. 7. AVR2 recognition is mediated by both *Cf-2.1* and *Cf-2.2*. Cf0 tomato plants and Cf0 tomato plants transformed with either *Cf-2.1* or *Cf-2.2* (Dixon *et al.*, 1996) were inoculated with PVX::*Avr2*. Plants were photographed 14 days after inoculation with the recombinant virus.

The *Rcr3* gene is specifically required for *Cf-2*-mediated resistance (Dixon *et al.*, 2000). *Rcr3* mutants have been identified that either result in partial loss of *Cf-2*-mediated resistance (*rcr3-1*) or in a complete loss of *Cf-2*-mediated resistance (*rcr3-3*) (Dixon *et al.*, 2000). Inoculation of both rcr3 mutant Cf2 lines with PVX::*Avr2* did not result in visible HR symptoms at 14 days after inoculation (results not shown), whereas Cf2 plants with a functional *Rcr3* gene show severe necrosis at this time point (see Fig. 3). At 22 days after inoculation, however, chlorosis and slight necrosis was observed in rcr3-1 Cf2 plants

inoculated with PVX::*Avr2*, whereas only typical viral mosaic symptoms were observed in rcr3-3 Cf2 plants (Fig. 8). These experiments show that the ability of rcr3-mutant Cf2 plants to respond to AVR2 with a HR correlates with their degree of resistance to AVR2-producing strains of *C. fulvum*.

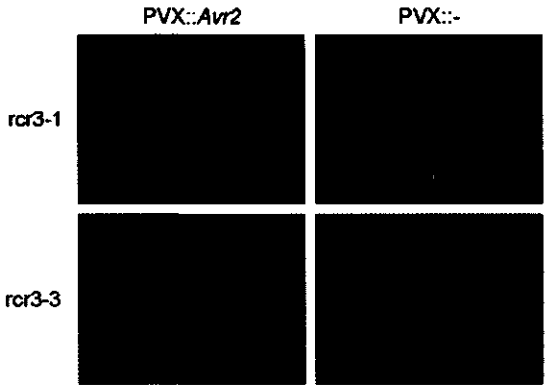


Fig. 8. The ability of two different rcr3-mutant Cf2 tomato lines to respond to AVR2 with a HR, correlates with their degree of resistance. Mutant Cf2 tomato plants that either show partial (rcr3-1) or a complete (rcr3-3) loss of *Cf-2*-mediated resistance, were inoculated with PVX::*Avr2* or PVX::-. Leaves were photographed at 22 days after PVX inoculation. Note that weak chlorosis and necrosis is only observed in rcr3-1 mutant plants that were inoculated with PVX::*Avr2*, whereas the other plants only show mosaic symptoms due to PVX infection.

Discussion

Cloning and characterisation of Avr2

Avr2 cDNA was cloned based on the specific HR-inducing activity of the encoded protein in Cf2 tomato plants, according to the method developed by Takken *et al.* (2000b). To our knowledge, this is the first novel *Avr* gene that has been cloned employing this strategy. The predicted AVR2 protein is a small, cysteine-rich, secreted protein (Fig.1). The latter is consistent with the presence of AVR2 protein in apoplastic fluid (De Wit and Spikman, 1982 and Fig. 5). In analogy with AVR4 and AVR9 (Van Kan *et al.*, 1991; Van den Ackerveken *et al.*, 1993b; Joosten *et al.*, 1994; Joosten *et al.*, 1997), the AVR2 protein might get further processed by plant and/or fungal proteases after cleavage of the predicted signal peptide. It was not possible, however, to determine the molecular weight of the AVR2 protein present in apoplastic fluid by western analysis, as attempts to raise antibodies against a synthetic peptide derived from AVR2, were not successful.

Strain #31 of *C. fulvum*, which is virulent on Cf2 plants due to insertion of a LINE-like element (Cfl1) in the *Avr2* ORF, was transformed with a genomic clone of *Avr2*. The *Avr2* ORF containing the Cfl1 insert is too long to be amplified with *Avr2*-specific primers using standard PCR conditions. As a result, PCR analysis of the transformants using standard conditions only revealed the presence of the *Avr2* transgene. Based on previous transformation experiments with *C. fulvum* (Van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994), the transformants are expected to contain multiple copies of the *Avr2* transgene. PCR analysis, using an *Avr2*-specific primer combined with a *Cfl1*-specific primer, revealed that all transformants still contain the *Cfl1* insert in the endogenous *Avr2* gene (R.

Luderer, unpublished results). The latter validates that the transformants have become avirulent on Cf2 plants due to incorporation of the *Avr2* transgene. These results show that AVR2 is not only inducing a HR in Cf2 tomato plants (Fig. 3), but that expression of *Avr2* actually confers avirulence of *C. fulvum* on Cf2 tomato plants (Fig. 4).

Various modifications were identified in the *Avr2* ORF of strains of *C. fulvum* that are virulent on Cf2 tomato plants. These modifications are all predicted to result in a truncated AVR2 protein (Table 1). The truncated AVR2 proteins all lack at least three cysteine residues, when compared to the predicted mature AVR2 protein. For AVR4 and AVR9 it has been shown that the substitution of one single cysteine residue severely compromises their HR-inducing activity of the protein (Joosten *et al.*, 1997; Kooman-Gersmann *et al.*, 1997). Moreover, it has been shown that the cysteine residues of AVR9 are involved in disulfide bridges, which are important for the structure of the protein (Van den Hooven *et al.*, 2001). It is therefore not surprising that the truncated AVR2 proteins are not functional as an AVR protein.

Beside their function as avirulence protein, AVR proteins are thought to contribute to virulence of the pathogen as well (Gabriel, 1999; Kjemtrup *et al.*, 2000; White *et al.*, 2000; Van 't Slot and Knogge, 2002). The truncated AVR2 proteins are probably also compromised in their potential virulence function. Considering the large number of independent, different *Avr2* mutations identified in our world-wide *C. fulvum* collection, the AVR2 protein apparently is not crucial for virulence of *C. fulvum*. This is consistent with the fact that we do not observe visible differences between growth of *C. fulvum Avr2*-transformants and the recipient strain on Cf0 tomato plants. The *Avr9* gene is absent in strains of *C. fulvum* that are virulent on Cf9 plants, indicating that AVR9 is dispensable for virulence of *C. fulvum* as well (Van Kan *et al.*, 1991). This was confirmed under laboratory conditions by disrupting the *Avr9* gene in *C. fulvum* (Marmeisse *et al.*, 1993). Strains of *C. fulvum* that are virulent on Cf4 plants contain mutations in the *Avr4* ORF (Joosten *et al.*, 1994; Joosten *et al.*, 1997). In contrast to the mutations identified in *Avr2*, the mutations identified in *Avr4*, except for one, result in single aa substitutions (Joosten *et al.*, 1994; Joosten *et al.*, 1997). Although the avirulence function of these AVR4 isoforms is affected, they might still contribute to the virulence of the fungus. From this point of view, the modifications identified in the *Avr2* ORF are intrinsically different from most of the modifications identified in the *Avr4* ORF. Whether the large number of mutations in *Avr2* are the result of the dispensability of AVR2 for virulence of the fungus or that they are caused by a severe selection pressure by tomato plants carrying the *Cf-2* resistance gene can not be concluded, as for most of the strains in our collection it is not known under which conditions they have been isolated. Beside race-specific avirulence proteins, *C. fulvum* secretes several other proteins, such as ECP1, ECP2, ECP3, ECP4 and ECP5 (for extracellular proteins), that have been shown to possess elicitor activity on specific tomato genotypes (Haanstra *et al.*, 2000; Laugé *et al.*, 2000). For ECP1, ECP2, ECP4 and ECP5 the encoding gene has been cloned, revealing that all ECPs, like the AVR proteins, are small, cysteine-rich, secreted proteins (Van den Ackerveken *et al.*, 1993a; Laugé *et al.*,

2000). As the ECP proteins are produced by all strains of *C. fulvum* analysed so far, they are thought to be virulence factors of the fungus. The latter was confirmed for ECP1 and ECP2 by analysis of *C. fulvum* mutants in which the encoding genes were disrupted (Laugé *et al.*, 1997). On the other hand, the absence of modifications in *Ecp* genes might reflect a lack of selection pressure to overcome *Cf-ECP*-mediated resistance.

Cfl1, a LINE-like element of C. fulvum

Two strains of *C. fulvum* present in our collection contain a Cfl1 insertion in their *Avr2* ORF (Fig 6). Cfl1 is the first LINE-like element (also known as non-LTR retrotransposable elements) identified in *C. fulvum*. Cfl1 is located at the same position in the *Avr2* ORF in both strains, with an identical target site duplication. These results indicate that the two strains are related, which is consistent with the fact that they were both isolated on the North American continent. Both strains produce all known elicitor proteins except for AVR2, however, they are different with respect to growth rate and colour of their spores. As a sexual stage has never been observed for *C. fulvum*, the action of transposable elements is thought to contribute to the genetic variation within the *C. fulvum* population. Whether the identified Cfl1 element of *C. fulvum* is still mobile remains to be determined.

LINE-like elements can make up a considerable part of an eukaryotic genome. For example, the L1 LINE-like element accounts for about 15% of the human genome sequence (Kazazian and Moran, 1998). Southern blot analysis of strains of *C. fulvum* isolated world-wide has indicated that the *C. fulvum* genome contains multiple copies of Cfl1, irrespective of the location where the strain was isolated (R. Luderer, unpublished results). AFLP analysis of our world-wide *C. fulvum* collection did not reveal sufficient polymorphisms for a reliable phylogenetic characterisation, as the *C. fulvum* population seems to consist of a single clonal lineage (Joosten and De Wit, 1999). Considering the general presence of Cfl1 elements in *C. fulvum*, Cfl1 polymorphisms within the *C. fulvum* population might be employed for a more detailed phylogenetic study. A similar approach was proven to be successful for *Fusarium oxysporum* employing the SINE element *Foxy* (Mes *et al.*, 2000).

To our knowledge, this is the first report of gain of virulence of a plant pathogen towards a specific host genotype due to insertion of a retrotransposable element in an *Avr* gene. As mentioned above, circumvention of recognition of AVR proteins has been reported to occur by absence of the *Avr* gene, by point mutations in the *Avr* gene and by frame-shift mutations (reviewed by Van 't Slot and Knogge, 2002). Furthermore, insertion of mobile elements that transpose at the DNA level, either in the ORF of an *Avr* gene or in its promoter, has been reported to result in gain of virulence of plant pathogens towards specific host genotypes (Kearney and Staskawicz, 1990a; Kang *et al.*, 2001). These mechanisms to circumvent recognition by a resistant host can potentially occur in both prokaryotic and eukaryotic plant pathogens, however, retrotransposable elements, including LINE-like elements, are restricted to eukaryots.

Specific recognition of AVR2 by Cf2 plants

Our results demonstrate that AVR2 recognition is mediated by each of the *Cf-2* genes (Fig. 7). Furthermore, we revealed a correlation between the ability of two different rcr3-mutant Cf2 tomato lines to respond to AVR2 with a HR and their degree of *Cf-2*-based resistance (Fig. 8). The next challenge will be to determine the role of the extracellular Rcr3 protein in perception of AVR2 by Cf-2. We are currently employing the yeast *Pichia pastoris* to produce AVR2 protein that will be used to perform binding studies with both Cf-2 and/or Rcr3. These experiments should reveal whether Rcr3 represents the third component that is involved in perception of AVR2 by Cf-2. As suggested by Dixon *et al.* (2000), Rcr3 might represent the virulence target of AVR2 that is guarded by the Cf-2 protein. Considering its predicted protease activity, Rcr3 might be part of a basic defence mechanism of the plant that is inhibited by AVR2. However, Rcr3 might also be involved in modification of either AVR2 or Cf-2 to allow the specific recognition of the elicitor to occur. With the cloning of *Avr2*, all three components have become available to allow the dissection of the biochemical mechanism that is underlying the perception of AVR2 by Cf-2. These experiments can reveal important insights in the perception of AVR proteins by *R* gene products.

Experimental procedures

Fungal and plant material

C. fulvum strains were subcultured on potato-dextrose agar at 22 °C. Conidia from 10-day-old cultures were used for plant inoculations as described by De Wit (1977). Transformation of *C. fulvum* was performed as described by Van den Ackerveken *et al.* (1992). *C. fulvum* was co-transformed with pAN7-1 (Punt *et al.*, 1987) that contains a hygromycin resistance gene, to allow selection of transformants. For DNA isolation, *C. fulvum* was grown in liquid B5 medium as described by De Wit and Roseboom (1980).

Near-isogenic lines of tomato cultivar Moneymaker were grown in the greenhouse in a day-night regime of 16 h, 21°C light and 8 h, 19°C dark at 60% relative humidity. The Moneymaker genotype carrying no known *C. fulvum* resistance genes is referred to as Cf0 tomato, whereas Cf2 tomato refers to the near isogenic line carrying the *Cf-2* resistance locus. Other Moneymaker genotypes are also designated by the resistance gene they carry.

Functional screening of a cDNA library of C. fulvum

A cDNA library of strain 5a of *C. fulvum* (Takken *et al.*, 2000b), consisting of 9600 *Agrobacterium tumefaciens* colonies each containing a binary PVX-based vector with a cDNA insert, was toothpick-inoculated on Cf2 and Cf9 tomato plants according to the procedure described by Takken *et al.* (2000b). The prudent selected 61 putative positives identified by the first screen, were rescreened on various tomato genotypes. From two clones, which reproducibly induced a specific HR upon inoculation onto Cf2 tomato plants, the insert sequence was obtained according to the procedure described by Takken *et al.* (2000b).

PCR analysis

For standard PCR analysis, *C. fulvum* DNA was isolated according to the procedure of Cenis (1992) after growth of the fungus for 5 days in liquid B5 medium. After DNA isolation, the *Avr2* ORF was PCR-amplified (45 sec 94 °C, 45 sec 60 °C, 1 min 72 °C, 30 cycles) using *AmpliTaq* DNA polymerase (Perkin Elmer) with primers AVR2F (5'-CTCCGCCCAACATTCGAC-3') and AVR2R (5'-CTCTTCTCACACTGTCTCC-3'), which are flanking the *Avr2* ORF. The PCR products were sequenced using the AVR2R primer.

For PCR analysis optimised for obtaining long PCR products, *C. fulvum* DNA was isolated by grinding freeze-dried mycelium obtained from a liquid culture in an eppendorf tube. After homogenisation in 1 ml extraction buffer (50 mM EDTA, 0.2% SDS (pH 8.5)), 10 µl proteinase K (10 mg/ml) were added and the mixture was incubated for 15 min at 70 °C. Subsequently, 100 µl 5 M KAc were added and the mixture was left on ice for 30 min. After centrifugation (15 min, 15,600 x g), the supernatant was treated with RNAse (1 µl (10 mg/ml), for 30 min at 37 °C), extracted twice with phenol:chloroform:isoamylalcohol (25:24:1) and once with chloroform:isoamylalcohol (24:1). After extraction, 1/10 volume 3M NaAc and ½ volume 7.5 M NH_4Ac were added to the upper phase and the mixture was left on ice for 15 min. After addition of 1 volume of isopropanol, the mixture was incubated for 5 min at room temperature and centrifuged (15 min, 15,600 x g). The precipitated DNA was washed with 70% EtOH and resuspended in TE (10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)).

This DNA (12 ng) was subsequently used for PCR using Expand polymerase mixture (Boehringer Mannheim), according to the protocol of the manufacturer. Amplification (10 cycles of 15 sec 95 °C, 45 sec 60 °C, 8 min 68 °C, followed by 20 cycles with an addition of 5 sec extension (68°C) time per cycle) with AVR2F and AVR2R resulted for strain #31 and #41 in a fragment of about 5.3 kb, that was inserted into the pGEM-T easy vector (Promega). Initially, the fragments were sequenced with AVR2F and AVR2R, resulting in a partial sequence. The 5.3 kb fragment derived from strain #31 was sequenced completely following a primer-walking approach (Baseclear, Leiden, The Netherlands).

Screening of a genomic library of C. fulvum

The *Avr2* cDNA sequence was amplified with AVR2F and AVR2R using standard PCR conditions. The resulting 334-bp *Avr2* fragment was used as a probe to screen a λBlueStar (Novagen) genomic library of *C. fulvum*. Eight independent clones, ranging from about 12 kb till over 20 kb, were identified that contained a full-length *Avr2* ORF. The position of the *Avr2* ORF in these clones was estimated by PCR optimised for long PCR products (see above) with vector-specific and *Avr2*-specific primers. Two clones containing about 10 kb and 11 kb at the 5'-end of the *Avr2* ORF and both 4,5 kb at the 3'-end of the *Avr2* ORF, were used for transformation of *C. fulvum*.

RNA isolation and Northern blotting

Total RNA was isolated from *C. fulvum*-infected tomato leaves according to the hot-phenol procedure (Extract-A-Plant RNA isolation kit, Clontech). From each sample, 15 µg glyoxal-denatured RNA was separated on a 1.4% agarose gel and transferred to Hybond-N^+ membrane (Amersham). Prehybridisation and hybridisation with ^{32}P-labeled probes was performed in a phosphate buffer modified after Church and Gilbert (1984) (0.5 M phosphate buffer (pH 7.2), 7% SDS, 1mM EDTA) at 65 °C. Blots were washed (0.5 x SSC, 0.5% SDS, 65 °C) and X-omat AR film (Kodak) was exposed to the blots at –80 °C.

*Construction of PVX::*Avr2, in vitro *transcription and plant inoculation*

For transient expression of *Avr2 in planta*, recombinant potato virus X (PVX) expressing *Avr2* (PVX::*Avr2*) was constructed. The *Avr2* cDNA was PCR-amplified (1 min 94 °C, 1 min 50 °C, 2 min 72 °C, 30 cycles) with *Pfu* DNA polymerase (Stratagene), using primers that introduce a *Cla*I restriction site (underlined) 5' of the start codon (5'-ATGCAATCGATATGAAGCTCTTCATACTGACC-3') and 3' of the stopcodon (5'-TACGTATCGATTCATCAACCGCAAAGACC-3') of the *Avr2* ORF. The resulting PCR fragment was digested with *Cla*I and ligated into *Cla*I-digested, dephosphorylated PVX plasmid vector (Chapman *et al.*, 1992b), 3' of the duplicated coat protein promoter. The sequence of PVX::*Avr2* was verified and a correct construct was used for in *vitro* transcription and inoculation of three to four week-old tomato plants, as described by Kooman-Gersmann *et al.* (1997).

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Chapter 5

Avirulence proteins of plant pathogens: determinants of victory and defeat

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Abstract

The simplest way to explain the biochemical basis of the gene-for-gene concept is by direct interaction between a pathogen-derived avirulence (*Avr*) gene product and a receptor protein, which is encoded by the matching resistance (*R*) gene of the host plant. The number of *R* genes for which the matching *Avr* gene has been cloned is increasing. The number of host-pathogen relationships, however, for which a direct interaction between *R* and *Avr* gene products could be proven is still very limited. This observation suggests that in various host-pathogen relationships no physical interaction between R and Avr proteins occurs, and that perception of AVR proteins by their matching *R* gene products is indirect. Indirect perception implies that at least a third component is required. The 'Guard hypothesis' proposes that this third component could be the virulence target of an AVR protein. Binding of the AVR protein to its virulence target is perceived by the matching R protein, which is 'guarding' the virulence target. An intriguing aspect of the 'Guard hypothesis' is that the *Avr* gene product causes avirulence of the pathogen through interaction with its virulence target in the plant. This would mean that, although AVR proteins are generally thought to be bifunctional (avirulence as well as virulence factors), this dual function might be based on a single biochemical event. This review focuses on the way AVR proteins are perceived by their matching *R* gene products. The various components that determine the outcome of the interaction will be discussed, with an emphasis on the dual function of AVR proteins.

Introduction

The gene-for-gene model postulates that for every dominant gene determining resistance in the host plant, there is a matching dominant gene conditioning avirulence in the pathogen (Flor, 1942, 1946; Oort, 1944). The prevailing model for explaining the biochemical basis of the gene-for-gene concept is the elicitor-receptor model (Gabriel and Rolfe, 1990; Keen, 1990). According to this model, an avirulence (*Avr*) gene of a pathogen encodes an elicitor protein that is perceived by a receptor protein, which is encoded by the matching resistance (*R*) gene of the host plant. The simplest mode of elicitor perception by a resistant host would be a direct interaction of the pathogen-derived elicitor with the matching resistance gene product. Perception of the elicitor protein by the host plant subsequently leads to the activation of defence responses, often including cell death around the infection site (Hammond-Kosack and Jones, 1996). The latter is referred to as the hypersensitive response (HR) and is a hallmark of gene-for-gene based resistance.

Features of R and Avr proteins

To date, a variety of *R* and *Avr* genes have been cloned [Reviewed by Takken and Joosten (2000a) and Van 't Slot and Knogge (2002)]. The encoded R proteins are remarkably similar and can be classified based on their structural domains and their localisation

(Takken and Joosten, 2000a). Consistent with their predicted receptor function all R proteins, except for Pto and RPW8, contain a leucine-rich repeat (LRR) domain which is thought to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). The *R* genes can be divided into those encoding proteins with cytoplasmic LRRs and those encoding proteins with extracytoplasmic LRRs (Jones and Jones, 1996). The R proteins with a cytoplasmic LRR domain all possess a nucleotide-binding site (NBS). Furthermore, some of these R proteins contain a leucine zipper (LZ) domain or a Toll/interleukin 1 receptor (TIR) domain. The R proteins with an extracytoplasmic LRR domain contain a transmembrane region and one of them, Xa21, also contains a cytoplasmic protein kinase domain (Song *et al.*, 1995; Takken and Joosten, 2000a). The structural domains of R proteins predict a role in signal transduction. In most cases, however, it is not clear how R proteins initiate defence responses.

In contrast to the R proteins, the *Avr* gene products identified so far show little obvious similarity (Van 't Slot and Knogge, 2002). The fact that most *Avr* genes are maintained within a pathogen population suggests that their products, in addition to their role as avirulence factor, have a function that is beneficial for the pathogen. Although the number of *Avr* genes for which a clear function for the pathogen has been demonstrated is still limited, it is now generally accepted that *Avr* gene products are bifunctional and also have a role in the virulence of the pathogen (Gabriel, 1999; Kjemtrup *et al.*, 2000; Van 't Slot and Knogge, 2002; White *et al.*, 2000). AVR proteins might contribute to pathogen virulence through interaction with specific plant proteins, so-called virulence targets. Interaction of AVR proteins with virulence targets that are, for example, involved in host metabolism or in plant defence, could result in enhanced nutrient availability for the pathogen or a suppression of defence responses, respectively (Van der Biezen and Jones, 1998).

Perception of AVR proteins

The number of *R* genes for which the matching *Avr* gene has been cloned is increasing. The number of host-pathogen relationships, however, for which a direct interaction between *R* and *Avr* gene products has been detected, is still very limited (see below). This suggests that in many host-pathogen relationships no physical interaction between R and Avr proteins occurs and that the perception of AVR proteins by their matching *R* gene products is indirect. Indirect perception of AVR proteins by R proteins implies that at least a third component is required for specific recognition of an avirulence factor by a resistant host. As indirect perception of an AVR protein depends on its binding to a putative third component, a correlation is expected between the binding affinity of the AVR protein for the third component and its HR-inducing activity. Although the nature of the third component is speculative, it might for example be a co-receptor. Another fascinating possibility is the 'Guard hypothesis' that was put forward by Van der Biezen and Jones (1998). The 'Guard hypothesis' proposes that the third component that is required for perception of an AVR protein is represented by the virulence target of the AVR protein. Binding of the AVR protein to its virulence target is perceived by the matching R protein, which is 'guarding'

this virulence target. An intriguing aspect of the 'Guard hypothesis' is that it predicts that the *Avr* gene product causes avirulence by interaction with its virulence target. This would mean that, although AVR proteins are generally regarded to be bifunctional, this dual function might be based on a single biochemical event, funnelling into two different pathways. In the presence of the 'guarding' R protein, binding of the AVR protein to its virulence target will result in the initiation of defence responses (defeat), whereas in case of absence of the R protein binding will result in enhanced susceptibility (victory). The latter implies that, if the third component indeed represents a virulence target, a correlation is expected between the HR-inducing activity of the AVR protein and its contribution to virulence.

This review focuses on the way AVR proteins are perceived by their matching *R* gene products. For this purpose, only gene-for-gene relationships were selected for which both the *Avr* and the *R* gene have been cloned (listed in Table 1). The components that determine the outcome of the interaction will be discussed, with an emphasis on the dual function of AVR proteins.

Bacterial AVR Proteins

AvrBs2 of X. campestris

The *Bs2* gene of pepper confers resistance to strains of *Xanthomonas campestris* pv. *vesicatoria* expressing *avrBs2* (Minsavage *et al.*, 1990). *Bs2* encodes an NBS-LRR protein with a hydrophobic N-terminus (Tai *et al.*, 1999). The *avrBs2* gene is needed for full virulence of the bacterium on susceptible hosts and it was shown to be highly conserved among different strains of *X. campestris* pv. *vesicatoria* and among other pathovars of *X. campestris* (Kearney and Staskawicz, 1990b). The *avrBs2* gene encodes a predicted protein of 80.1 kD, which is mainly hydrophilic (Swords *et al.*, 1996). The C-terminal half of the predicted AvrBs2 protein has homology with enzymes that synthesise or hydrolyse phosphodiester linkages (Swords *et al.*, 1996). To date, however, it is not known whether this homology is relevant for the virulence function of AvrBs2. Initially, all *avrBs2* mutants that were unable to trigger an *avrBs2*-specific HR displayed reduced fitness in susceptible cultivars (Swords *et al.*, 1996). This correlation between HR inducing activity and contribution to virulence could be an indication for indirect perception of AvrBs2 by Bs2 after binding of the AvrBs2 protein to its virulence target. Recently, however, an *avrBs2* mutant was identified, *avrBs2-3*, that showed hardly any reduction in fitness on susceptible plants, without triggering a resistance response on plants carrying *Bs2* (Gassmann *et al.*, 2000). As this observation seems to uncouple the virulence and the avirulence function of AvrBs2, specific perception of AvrBs2 is unlikely to occur after binding to its virulence target. Identification of the virulence target will be essential to confirm this conclusion.

Table 1. Gene-for-gene relationships discussed in this review. The gene-for-gene pairs are listed in the order by which they appear in the text. For references see text.

Pathogen	AVR protein	Virulence function	Third component	R protein	Host
X. campestris pv. *vesicatoria*	AvrBs2	Required for full virulence	?	Bs2	Pepper
P. syringae pv. *tomato*	AvrPto	Enhances virulence in tomato	Pto	Prf	Tomato
P. syringae pv. *phaseolicola*	AvrPphB	Weak browning upon expression in bean	PBS1	RPS5	*Arabidopsis*
P. syringae pv. *pisi*	AvrRps4	Unknown	?	RPS4	*Arabidopsis*
P. syringae pv. *tomato*	AvrRpt2	Promotes virulence	p75	RPS2	*Arabidopsis*
P. syringae pv. *maculicola*	AvrRpm1	Required for virulence on *Arabidopsis*	RIN4	RPM1	*Arabidopsis*
P. syringae pv. *glycinea*	AvrB	Triggers chlorosis	RIN4	RPM1	*Arabidopsis*
M. grisea	AVR-Pita	Predicted zinc metalloprotease	No	Pi-ta	Rice
C. fulvum	AVR2	Unknown	Rcr3	Cf-2	Tomato
C. fulvum	AVR4	Unknown	?	Cf-4	Tomato
C. fulvum	AVR9	Unknown	HABS	Cf-9	Tomato
Potato Virus X	CP	Coat protein	?	Rx1, Rx2	Potato
Turnip crinkle virus	CP	Coat protein	TIP	HRT	*Arabidopsis*
Tobacco mosaic virus	Replicase	Replicase/helicase	?	N	Tobacco

AvrPto of P. syringae

The avirulence gene *avrPto* of *Pseudomonas syringae* pv. *tomato* confers avirulence of the bacterium on tomato cultivars carrying the *Pto* gene (Ronald *et al.*, 1992). AvrPto is a hydrophilic protein of 164 amino acids and was shown to enhance virulence in tomato lines lacking *Pto* (Chang *et al.*, 2000; Salmeron and Staskawicz, 1993). A direct, physical interaction was reported between AvrPto and Pto and a strong correlation was found between the presence of an interaction between mutants of these proteins in the yeast two-hybrid system and the ability to elicit a HR *in planta* (Scofield *et al.*, 1996; Tang *et al.*, 1996). These experiments were considered as the first evidence of direct perception of an AVR protein by a plant R protein. The *Pto* gene encodes a protein kinase (Martin *et al.*, 1993) and lacks the structural features, like an LRR domain, that are shared by other R proteins. Interestingly, for *avrPto/Pto*-mediated resistance, *Prf*, a gene encoding a cytoplasmic R-like LZ-NBS-LRR protein, is also required (Salmeron *et al.*, 1996). To explain the role of Prf in *avrPto/Pto*-mediated signalling, Van der Biezen and Jones (1998) launched the 'Guard hypothesis' and proposed that *Pto* is not the true *R* gene, but encodes the virulence target of AvrPto. Pto is involved in the regulation of basic defence responses (Tang *et al.*, 1999), which might be repressed following the binding of AvrPto to Pto (Van der Biezen and Jones, 1998). The AvrPto-Pto complex is subsequently perceived by the true R protein, Prf, which is 'guarding' the virulence target. Mutants of AvrPto that lack avirulence activity, however, are not always impaired in their virulence function (Shan *et al.*, 2000). This might indicate that Pto is not a virulence target of AvrPto or that AvrPto

might have multiple virulence targets. The latter is consistent with the data of Bogdanove and Martin (2000) who have shown, employing the yeast two-hybrid system, that AvrPto interacts with several plant proteins that each represent potential virulence targets. Thus, the data currently available are more consistent with an indirect perception of AvrPto by Prf, instead of a direct perception of AvrPto by Pto. Therefore the interaction between AvrPto and Pto should not be considered as an example of direct interaction between an *Avr* and an *R* gene product but rather as interaction between an AVR protein and its virulence target.

AvrPphB of P. syringae

The *avrPphB* gene (originally described as *avrPph3*) of *Pseudomonas syringae* pv *phaseolicola* can confer avirulence of the bacterium on bean, pea, soybean and *Arabidopsis* (Fillingham *et al.*, 1992; Jenner *et al.*, 1991; Pirhonen *et al.*, 1996; Simonich and Innes, 1995). AvrPphB is a hydrophilic protein, which is N-terminally processed, resulting in a 28 kD peptide that contains a myristoylation motif (Puri *et al.*, 1997). *Agrobacterium tumefaciens*-mediated expression of *avrPphB* in bean results in a HR in cultivars carrying the *R3* resistance gene, whereas in some bean cultivars lacking *R3*, a weak browning was induced upon *avrPphB* expression (Stevens *et al.*, 1998). For perception of AvrPphB by *Arabidopsis*, at least two genes, *RPS5* and *PBS1* are required. *RPS5* encodes a LZ-NBS-LRR protein, featuring the typical structural domains of R proteins (Warren *et al.*, 1998). *PBS1* encodes a functional protein kinase with similarity to the Pto kinase of tomato (Swiderski and Innes, 2001). It was therefore suggested that, in analogy with the perception of AvrPto by Pto and Prf, PBS1 might be the virulence target of AvrPphB, which is guarded by RPS5 (Swiderski and Innes, 2001). Interestingly, RPS5, PBS1 and AvrPphB all contain a myristoylation motif, suggesting a common localisation of the three proteins at the plasma-membrane. No interaction, however, could be detected between AvrPphB and PBS1, AvrPphB and RPS5, or PBS1 and RPS5 using yeast two-hybrid approaches (Swiderski and Innes, 2001). Further binding experiments might reveal whether AvrPphB binds to either PBS1 or RPS5 and should give more insight in whether perception of AvrPphB by RPS5 is indirect or not.

AvrRps4 of P. syringae

The *avrRps4* gene of *Pseudomonas syringae* pv. *pisi* can confer avirulence on *Arabidopsis* and soybean (Hinsch and Staskawicz, 1996). The predicted AvrRps4 protein has a molecular mass of 24 kD and is hydrophilic. All tested strains of *P. syringae* pv. *pisi* contain *avrPs4*-hybridising sequences (Hinsch and Staskawicz, 1996), suggesting that the gene might be important for virulence of the pathogen. Recognition of AvrRps4 in *Arabidopsis* is mediated by the *RPS4* gene. *RPS4* encodes a TIR-NBS-LRR protein, which is predicted to be cytosolic (Gassmann *et al.*, 1999). The availability of both the *Avr* and the *R* gene, encoding the components that determine the outcome of this gene-for-gene relationship, allows more detailed studies about the mechanism of perception of AvrRps4 by RPS4.

AvrRpt2 of P. syringae

The *Arabidopsis R* gene *RPS2* confers resistance to strains of *Pseudomonas syringae* pv. *tomato* carrying *avrRpt2* (Kunkel *et al.*, 1993; Innes *et al.*, 1993). RPS2 is a cytoplasmic LZ-NBS-LRR protein (Bent *et al.*, 1994; Mindrinos *et al.*, 1994). The *avr*Rpt2 gene encodes a protein of 28.2 kD, which is secreted and processed during bacterial pathogenesis (Innes *et al.*, 1993; Mudgett and Staskawicz, 1999). AvrRpt2 was shown to induce a HR in *Arabidopsis* plants carrying *RPS2*, whereas it was shown to promote virulence, resulting in increased growth of *P. syringae*, in the absence of a functional copy of *RPS2* in the host (McNellis *et al.*, 1998; Chen *et al.*, 2000). Co-immunoprecipitation experiments suggested an interaction of AvrRpt2 with a 75 kD plant protein and several additional plant proteins of 100 kD, which could indicate that AvrRpt2 has multiple virulence targets (Leister and Katagiri, 2000). A 75 kD protein was also found to immunoprecipitate with RPS2 and with RPS2 and AvrRpt2 together. It was anticipated that the same 75 kD protein, named p75, is co-immunoprecipitated in all cases. Based on the co-immunoprecipitation experiments AvrRpt2, RPS2 and p75 are thought to be present in one complex. It was therefore suggested that AvrRpt2 might be indirectly perceived by RPS2, after binding to p75 (Leister and Katagiri, 2000). Whether p75 is a virulence target of AvrRpt2 is not clear from their experiments. Banerjee *et al.* (2001) showed that effective interaction between RPS2 and at least one additional host factor is required for RPS2/AvrRpt2-mediated resistance. Although experimental evidence is not available, this factor might be represented by the p75 protein identified by Leister and Katagiri (2000). In summary, these data support the indirect perception of AvrRpt2 by RPS2. Identification of the nature of p75, will give more insight into the mechanism of perception of AvrRpt2.

AvrRpm1 and AvrB of P. syringae

The *avrRpm1* gene of *Pseudomonas syringae* pv *maculicola* confers avirulence of the bacterium on pea, bean, soybean and *Arabidopsis* (Dangl *et al.*, 1992). On the other hand, *avrRpm1* was shown to be required for virulence of *P. syringae* pv *maculicola* on *Arabidopsis* (Ritter and Dangl, 1995). Recognition of AvrRpm1 in *Arabidopsis* is mediated by *RPM1*. *RPM1* encodes a LZ-NBS-LRR peripheral membrane protein that is likely to reside at the cytoplasmic face of the plasma membrane (Boyes *et al.*, 1998; Grant *et al.*, 1995). Interestingly, *RPM1* also confers resistance to *Pseudomonas syringae* pv *glycinea* expressing *avrB* (Bisgrove *et al.*, 1994). Expression of *avrB* in *Arabidopsis* lines lacking *RPM1* revealed that AvrB is able to trigger chlorosis mediated by a slow cytotoxic response (Nimchuk *et al.*, 2000). The proteins encoded by *avrRpm1* and *avrB* do not share homology, except for an N-terminal eukaryotic consensus sequence for myristoylation and palmitoylation (Dangl *et al.*, 1992; Tamaki *et al.*, 1988). The myristoylation sequences of AvrRpm1 and AvrB are required for full avirulence function and for localisation of these proteins at the plasma membrane of the host plant (Nimchuk *et al.*, 2000). These data, together with the plasma membrane localisation of RPM1, are consistent with a membrane-

localised perception of AvrRpm1 and AvrB by RPM1. Dangl and co-workers recently showed that RIN4 (for RPM1-Interacting Protein 4), which is also probably localised at the plasma membrane, is required for the perception of both AvrRpm1 and AvrB by RPM1 (Eckardt *et al.*, 2001). In the absence of RPM1, AvrRpm1 and AvrB form a complex with RIN4. RIN4 is predicted to be their virulence target as it is a negative regulator of defence responses. These defence responses might be repressed following binding of AvrRpm1 and AvrB to RIN4. In uninfected tissue, RIN4 resides in a complex with RPM1. It was therefore concluded that the perception of AvrRpm1 and AvrB by RPM1 is indirect and that the third component that is required for perception is the virulence target RIN4 (Eckardt *et al.*, 2001).

Fungal AVR Proteins

AVR-Pita of M. grisea

The avirulence gene *AVR-Pita* of the rice blast fungus, *Magnaporthe grisea*, confers avirulence on rice cultivars containing resistance gene *Pi-ta*. *AVR-Pita* encodes a predicted 223-amino acid pro-protein, which is processed into a putative 176-amino acid mature protein that has homology to zinc-dependent metalloproteases (Jia *et al.*, 2000; Orbach *et al.*, 2000). *Pi-ta* encodes a predicted cytoplasmic protein containing a NBS domain and a leucine-rich carboxyl terminus (Bryan *et al.*, 2000). Employing the yeast two-hybrid system and *in vitro* binding assays, direct interaction was detected between the putative mature Avr-Pita protein and the leucine-rich domain of Pi-ta (Jia *et al.*, 2000). This finding is the first experimental evidence that is consistent with the model that AVR proteins directly interact with R proteins. Mutations in *Avr-Pita* or in *Pi-ta* that disrupted interaction in the yeast two-hybrid system also resulted in loss of the ability to induce a HR in the plant (Jia *et al.*, 2000). Interestingly, changes in the putative protease motif of AVR-Pita eliminated its avirulence function, indicating that the predicted protease activity of AVR-Pita is required for its avirulence function (Jia *et al.*, 2000). The latter is supported by the finding that for *in vitro* binding, zinc was required for refolding of AVR-Pita (Jia *et al.*, 2000). It will be intriguing to unravel the relationship between the putative protease activity of AVR-Pita and the ability to induce Pi-ta-dependent defence responses.

AVR2 of C. fulvum

Strains of the fungus *Cladosporium fulvum* carrying *Avr2* are avirulent on tomato plants carrying the *Cf-2* resistance locus. *Avr2* encodes a cysteine-rich protein of 78 amino acids, with a predicted signal peptide of 20 amino acids for extracellular targeting (R. Luderer, unpublished data). AVR2 is secreted by *C. fulvum* during growth in the apoplastic space of tomato leaves. Thus far, no virulence function has been assigned to AVR2, as natural strains of *C. fulvum* lacking a functional copy of *Avr2* that were complemented with a genomic clone of *Avr2*, did not show enhanced virulence on susceptible tomato lines (R. Luderer, unpublished data). The *Cf-2* resistance locus contains two functional resistance

genes, *Cf-2.1* and *Cf-2.2*, which are almost identical (Dixon *et al.*, 1996). Both genes encode a protein with a predicted signal peptide, an extracellular LRR region, a transmembrane domain and a short cytoplasmic tail with no homology to known signalling domains (Dixon *et al.*, 1996). The predicted extracellular localisation of the LRR region of the Cf-2 proteins, together with the presence of AVR2 in the apoplastic space in tomato leaves, is consistent with the extracellular perception of AVR2 by Cf-2. The *Rcr3* gene (for required for *C. fulvum* resistance 3) was found to be specifically required for *Cf-2*-mediated resistance but not for *Cf-9*- or *Cf-5*-mediated resistance (Dixon *et al.*, 2000). As Cf-5 is over 90% identical to Cf-2 and their encoding genes map to the same position on chromosome 6, these *R* gene products are thought to activate the same defence signalling pathway following elicitor perception (Dixon *et al.*, 1998). Dixon *et al.* (2000) proposed that Rcr3 plays a role upstream of this common pathway and might represent the third component that is required for perception of AVR2 by Cf-2. To allow binding of AVR2 to Rcr3, Rcr3 is expected to be at least partially extracellular.

AVR4 of C. fulvum

The *Avr4* gene of *C. fulvum* confers avirulence on tomato lines carrying resistance gene *Cf-4*. *Avr4* encodes a 135-amino acid pre-pro-protein, which upon secretion is N- and C-terminally processed, resulting in an 86-amino acid mature AVR4 protein (Joosten *et al.*, 1994; Joosten *et al.*, 1997). The expression of *Avr4* is strongly induced at the onset of infection and AVR4 is secreted by the fungus in the apoplastic space of tomato leaves (Joosten *et al.*, 1997). Although the timing of expression suggests a role for AVR4 in the infection process, no virulence function has been reported for AVR4 thus far. Like *Cf-2*, the *Cf-4* gene encodes a predicted transmembrane protein with an extracellular LRR region and a short cytoplasmic tail (Thomas *et al.*, 1997). Cf-4 is predicted to be plasma membrane-localised, which is consistent with extracellular perception of AVR4 by Cf-4. Currently, De Wit and co-workers are unravelling the mechanism of perception of AVR4 by Cf-4.

AVR9 of C. fulvum

The *Cf-9* resistance gene of tomato confers resistance to strains of *C. fulvum* that carry the avirulence gene *Avr9*. *Cf-9* encodes a plasma membrane-localised protein that, similar to other Cf- proteins, contains an extracellular LRR region, a transmembrane domain and a short cytoplasmic tail with no homology to known signalling domains (Jones *et al.*, 1994; Piedras *et al.*, 2000). The AVR9 protein is secreted by *C. fulvum* in the apoplastic space of tomato leaves (Scholtens-Toma and De Wit, 1988). The *Avr9* gene encodes a precursor protein of 63 amino acids, which is further processed by fungal and plant proteases into a 28 amino acid peptide (Van den Ackerveken *et al.*, 1993b; Van Kan *et al.*, 1991). Disruption of the *Avr9* gene in *C. fulvum* did not reveal an apparent virulence function of AVR9 in laboratory experiments (Marmeisse *et al.*, 1993). Expression of *Avr9*, however, is induced under nitrogen-limiting conditions (Pérez-Garcia *et al.*, 2001; Van den Ackerveken *et al.*, 1994), suggesting a role for AVR9 in (nitrogen) metabolism of the fungus. Binding

studies with AVR9 and Cf-9 were performed in a collaborative, multidisciplinary approach of various research groups in Europe, combining the latest technology. Despite these extensive experiments, no specific binding between AVR9 and Cf-9 was detected (Luderer *et al.*, 2001b). Binding studies with radiolabeled AVR9 protein, however, revealed the presence of a high affinity binding site (HABS) in plasma membranes of tomato and other solanaceous plants (Kooman-Gersmann *et al.*, 1996). The binding affinity of mutant AVR9 peptides for the HABS was shown to correlate with their ability to induce a HR in tomato lines carrying *Cf-9* (Kooman-Gersmann *et al.*, 1998). Kooman-Gersmann *et al.* (1998) therefore suggested that the HABS might be a third component that is required for perception of AVR9 by Cf-9. This hypothesis is supported by their common localisation and by the fact that a Cf-9/AVR9-mediated HR could only be induced in species that were shown to contain the HABS (Hammond-Kosack *et al.*, 1998; Kooman-Gersmann *et al.*, 1996; Van der Hoorn *et al.*, 2000). A functional Cf-9/AVR9-mediated HR was reported to occur in *Brassica napus* (Hennin *et al.*, 2001), whereas the HABS was shown to be absent in another *Brassica* species, *B. oleracea* (Kooman-Gersmann *et al.*, 1996). Considering the high amount of AVR9 that was needed to induce a HR in *Cf-9*-transgenic *B. napus* (Hennin *et al.*, 2001), it is questionable, however, whether the observed necrosis represents a true, specific HR. As the HABS is present in plasma membranes from tomato and other solanaceous plants, this binding site probably has a conserved function and might represent the virulence target of AVR9. The latter suggestion, however, can only be confirmed by gaining more insight in the nature of the HABS.

Viral AVR Proteins

The coat protein of PVX

The coat protein (CP) of potato virus X (PVX) confers avirulence of the virus in potato lines carrying either *Rx1* or *Rx2* (Bendahmane *et al.*, 1995). *Rx1* and *Rx2* encode predicted cytoplasmic LZ-NBS-LRR proteins of which the C-terminal LRR region is almost identical (Bendahmane *et al.*, 1999; Bendahmane *et al.*, 2000). The *CP* gene encodes a 25 kD protein, which is essential for the production of virions and systemic spread of PVX in the plant (Chapman *et al.*, 1992a). Several CP mutants of PVX that were impaired in forming virions and could not spread in the plant, still showed at least partial elicitor activity in potato carrying *Rx* (Bendahmane *et al.*, 1995). These data indicate that the elicitor activity of the CP of PVX is independent from its function as a viral coat protein. A region between amino acid residues 32 and 139 of the CP was shown to be essential for elicitor activity (Bendahmane *et al.*, 1995). Whether this region establishes direct interaction with the Rx protein remains to be elucidated.

The coat protein of TCV

The *HRT* gene of *Arabidopsis* confers resistance to turnip crinkle virus (TCV) (Dempsey *et al.*, 1997). *HRT* encodes a LZ-NBS-LRR protein, which mediates recognition of the TCV coat protein (CP) (Cooley *et al.*, 2000). The CP is a 38 kD protein that packages the viral RNA. The N-terminus of the CP is involved in eliciting resistance responses (Zhao *et al.*, 2000). Interestingly, interaction between this CP and another protein, designated TIP (for TCV-interacting protein), is required for *HRT*-mediated resistance (Ren *et al.*, 2000). All CP mutants that did not interact with TIP in the yeast two-hybrid system, also lost the ability to induce an *HRT*-mediated resistance response, whereas the mutations did not affect the formation of virions. These results indicate that the evident function of the CP as the coat protein of TCV does not correlate with its ability to bind TIP and that the CP probably has multiple functions. The latter is not unusual for viral proteins. TIP is a putative transcription factor and Ren *et al.* (2000) therefore suggested that TIP is the virulence target of the CP. TIP might be involved in the regulation of basic defence responses, which could be repressed following binding of the CP (Ren *et al.*, 2000). The HRT protein is proposed to 'guard' TIP by detecting conformational changes in TIP due to interaction with the CP. Thus far, however, no reduced fitness was reported for TCV mutants producing a CP that is unable to bind to TIP. A more detailed analysis of the fitness of these mutants might reveal whether TIP indeed represents a virulence target of the CP of TCV.

The replicase of TMV

The replicase proteins of tobacco mosaic virus (TMV) confer avirulence of the virus on tobacco lines carrying the *N* gene (Padgett and Beachy, 1993; Padgett *et al.*, 1997). The *N* gene encodes a TIR-NBS-LRR protein, which is predicted to be cytoplasmic (Whitham *et al.*, 1994). The TMV genome encodes a 126 kD replicase protein and by read-through of the stop codon an additional 183 kD replicase protein is produced. A 50 kD region of these replicases that may function as a helicase is sufficient to induce a HR in tobacco carrying the *N* gene (Erickson *et al.*, 1999). The putative helicase function of the 50-kD fragment was confirmed by biochemical and mutational analysis. The helicase function, however, is not required for the avirulence function of the replicase proteins (Erickson *et al.*, 1999). Whether perception of the replicase protein by the N protein is direct or indirect remains to be determined.

Concluding Remarks

In this review, 14 gene-for-gene relationships have been discussed, for which both the *Avr* and the *R* gene have been cloned. For only one of these a direct interaction between the *Avr* and the *R* gene-product is supported by experimental data. For eight of the described gene-for-gene relationships the experimental evidence is more consistent with an indirect perception of the AVR protein, whereas for five of them the mechanism of perception remains elusive.

The number of gene-for-gene relationships of which the AVR protein is likely to be perceived indirectly is surprisingly high. The exact mechanism underlying the involvement of a third component in perception of an AVR protein by a matching R protein remains to be elucidated (Dangl and Jones, 2001). The R protein might be bound to the third component prior to presence of the Avr protein, as is probably the case for RPS2 and RPM1. In other gene-for-gene relationships, however, complex formation with the R protein could take place after binding of the AVR protein to the third component. Subsequently, defence responses are initiated by this complex of proteins or after dissociation of (an activated form of) the R protein from the complex.

The most obvious implication of indirect perception of an AVR protein by an R protein is that at least a third component is required to mediate resistance. This means that not all plant genes that have been identified based on their specific requirement for gene-for-gene based resistance need to be *R* genes. The *Pto* gene for example, was initially identified as the *R* gene that was required for Pto-mediated resistance. The data currently available indicate that *Prf* is more likely to be the true *R* gene, whereas Pto is predicted to be the third component that is required for perception of AvrPto by Prf. This theory is supported by the fact that, in contrast with other R proteins, Pto is lacking a LRR region (Martin *et al.*, 1993; Takken and Joosten, 2000a). Another R protein that does not contain a LRR region is RPW8 (Xiao *et al.*, 2001). *RPW8* might therefore encode a third component that is required for AVR protein perception, rather than a R protein (Xiao *et al.*, 2001). These findings should be taken into account while developing new strategies for the cloning of *R* genes.

A second implication of the indirect perception of AVR proteins by R proteins is that perception of the AVR protein depends on binding of the AVR protein to the third component. Therefore a correlation is expected between the binding affinity of AVR proteins for the third component and their HR-inducing activity. The affinity of AVR proteins for the third component might be employed for its identification, using for example, affinity chromatography or the yeast two-hybrid system. The high amount of gene-for-gene relationships for which perception seems to be indirect might explain why these approaches might not have been successful in directly identifying *R* gene products.

The nature of the third component that is required for perception of AvrPto, AvrPphB, AvrRpm1, AvrB and the coat protein of TCV, has been revealed. Strikingly, the nature of all these proteins, Pto, PBS1, RIN4 (for AvrRpm1 and AvrB) and TIP, respectively, is consistent with a putative role as virulence target for the AVR protein, as they all might be involved in the regulation of basic defence responses. In these cases, the mechanism of perception of the AVR proteins is therefore consistent with the 'Guard hypothesis'.

Although the gene-for-gene relationships discussed in this review represent only a small selection of the total amount of gene-for-gene relationships that have been described to date, the data indicate that perception of AVR proteins by R proteins is predominantly indirect. Furthermore, as far as experimental data are available, this indirect perception appears to be consistent with the 'Guard hypothesis'. The maintenance of certain *R* genes

over a large number of generations in a plant population, was also reasoned to be the result of resistance based on guarding (Van der Hoorn *et al.*, 2002). The 'Guard hypothesis' stresses the central role of AVR proteins and their virulence targets in the outcome of plant-pathogen interactions. In absence of the matching *R* gene, AVR proteins manipulate their virulence target(s) to promote pathogen virulence, whereas they confer avirulence in the presence of the *R* gene. The fact that victory and defeat of a pathogen are based on a single feature of the AVR protein, binding to its virulence target, is an intriguing insight into their mode of action. This insight can be employed for a renewed approach to unravel their function. Moreover, the requirement of a third component to establish gene-for-gene-based resistance has important consequences for future research on the molecular basis of the outcome of gene-for-gene interactions and for modern resistance breeding.

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Chapter 6

Summarising discussion

Dual function of AVRs and ECPs

The research described in this thesis is focussed on the characterisation of elicitor proteins of *Cladosporium fulvum* and their perception by resistant tomato plants. During colonisation, *C. fulvum* secretes several avirulence (AVR) and ECP (for extracellular protein) elicitor proteins in the apoplastic space of tomato leaves (reviewed by Joosten and De Wit, 1999). The AVR proteins are specifically produced by some strains of *C. fulvum*, whereas the ECP proteins are produced by all strains analysed so far (De Wit and Spikman, 1982; Laugé *et al.*, 2000; Laugé *et al.*, 1998b; Van den Ackerveken *et al.*, 1993a; Wubben *et al.*, 1994). The AVR proteins owe their name to the fact that they elicit defence responses in tomato plants carrying the matching *Cf* resistance gene, resulting in avirulence of the fungus and resistance of the plant (Chapter 4, Joosten *et al.*, 1994; Van den Ackerveken *et al.*, 1992). As the ECP proteins are produced by all *C. fulvum* strains, they are thought to be virulence factors of the fungus. This was confirmed for ECP1 and ECP2 by disruption of the encoding gene (Laugé *et al.*, 1997) and it is expected that the same holds for ECP3, ECP4 and ECP5. More recently, it has been shown that ECP1, ECP2, ECP3, ECP4 and ECP5 possess, beside their (putative) virulence function, elicitor activity in genotypes of different Lycopersicon species (Haanstra *et al.*, 2000; Laugé *et al.*, 2000; Laugé *et al.*, 1998b). This finding is consistent with the established notion that elicitor proteins are bifunctional and have a role in virulence of the pathogen as well (Gabriel, 1999; Kjemtrup *et al.*, 2000; Van 't Slot and Knogge, 2002; White *et al.*, 2000). Thus far, no clear virulence function could be assigned to AVR2, AVR4 or AVR9 under laboratory conditions (Chapter 4; Joosten *et al.*, 1997; Marmeisse *et al.*, 1993). However, these AVRs might contribute to fungal virulence under field conditions.

The role of cysteine residues of elicitor proteins

A striking feature of all elicitor proteins of *C. fulvum* is that the mature form contains an even number of cysteine residues (reviewed by Joosten and De Wit, 1999). In analogy with the cysteine residues of AVR9 (Van den Hooven *et al.*, 2001), the cysteine residues of other *C. fulvum* elicitor proteins are thought to be involved in disulfide bridges, which are essential for proper conformation and stability of the proteins. Considering the presence of the elicitor proteins in the apoplastic space of tomato plants, their stability is thought to be very important, as the apoplast is known to be rich in proteases (Van den Ackerveken *et al.*, 1993b). To find out whether the even number of cysteine residues is of general importance for the stability and HR-inducing activity of the various elicitor proteins of *C. fulvum*, a mutational analysis was performed with ECP1, ECP2 and ECP5 (Chapter 2). Not all cysteine residues proved to be essential for full HR-inducing activity of the ECP proteins. Furthermore, some of the cysteine residues do not seem to be involved in intramolecular disulfide bridges of the mature ECPs (Chapter 2). Although experimental evidence is not available, these cysteine residues might be involved in intermolecular disulfide bridges. Such bridges could either result in homodimers or in complexes with other proteins, such as the putative virulence targets of the ECPs.

Perception of AVR9 by Cf-9

Both the AVR proteins and the ECPs are secreted by *C. fulvum* in the apoplastic space of tomato leaves during colonisation. The leucine-rich repeat (LRR) regions of all Cf resistance proteins of which the encoding gene has been isolated, are predicted to be located on the outer surface of the plasma-membrane (reviewed by Joosten and De Wit, 1999). LRRs are thought to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994). The extracellular localisation of the LRR region of the Cf proteins is consistent with a direct, extracellular perception of the elicitor proteins by the matching Cf proteins. To confirm this hypothesis, binding studies were performed between AVR9 and Cf-9. Although extensive studies on a possible direct interaction between the two proteins were performed in a multidisciplinary collaboration, combining the latest technology of various research groups in Europe, no specific binding between AVR9 and Cf-9 could be detected (Chapter 3). This implies that at least a third interacting partner is involved in the perception of AVR9 by Cf-9. Kooman-Gersmann *et al.* (1996) detected a high affinity binding site (HABS) for AVR9 in tomato and other solanaceous species. The HABS might be the third component that is required for perception of AVR9 by Cf-9, as the affinity of mutant AVR9 peptides for the HABS correlates with their HR-inducing activity in tomato lines carrying *Cf-9* (Kooman-Gersmann *et al.*, 1998). This hypothesis is supported by the common localisation of Cf-9 and the HABS in the plasma-membrane. As the HABS is present in tomato and other solanaceous species (Kooman-Gersmann *et al.*, 1996), it is thought to have a conserved function and might represent the virulence target of AVR9. To validate this hypothesis, efforts are being made to purify the HABS and clone its encoding gene. We propose that perception of AVR9 by Cf-9 is mediated by the HABS. This model is depicted in figure 1. In Cf0 tomato plants (that do not contain Cf-9), AVR9 will bind to the HABS (Fig. 1A). This will also occur in Cf9 tomato plants (that do contain Cf-9) (Kooman-Gersmann *et al.*, 1996). We propose that Cf-9 perceives binding of AVR9 to the HABS, resulting in the activation of defence responses (see also Kooman-Gersmann *et al.*, 1998 and Joosten and De Wit, 1999). How defence responses are initiated is not clear, as Cf-9 itself does not contain any obvious signalling domain (Jones *et al.*, 1994). Defence responses might be initiated by a cytoplasmic signalling domain of the HABS that is activated in the presence of both AVR9 and Cf-9 (Fig. 1B). Alternatively, upon perception of binding of AVR9 to the HABS by Cf-9, defence responses are initiated via an additional, cytoplasmic transducer protein (Fig. 1C).

Perception of AVR2 by Cf-2

Three tomato genes have been identified that are required for full *Cf*-mediated resistance against *C. fulvum*. Two genes that are required for *C. fulvum* resistance, *Rcr1* and *Rcr2*, were shown to be required for full *Cf-9*-mediated resistance (Hammond-Kosack *et al.*, 1994). *Rcr3* was found to be specifically required for *Cf-2*-mediated resistance but not for *Cf-9*- or *Cf-5*-mediated resistance (Dixon *et al.*, 2000). Like *Cf-9*, *Cf-2* encodes a predicted

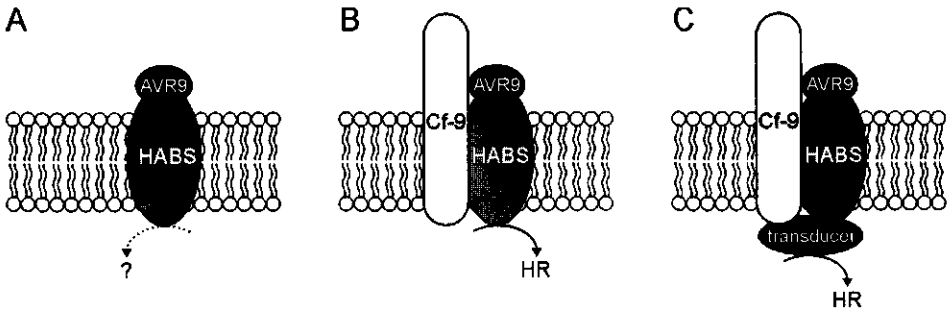

Fig. 1. Model for perception of AVR9 by tomato plants. (A) In Cf0 tomato plants (that do not contain Cf-9) AVR9 binds to the high affinity binding site (HABS), its putative virulence target. The dotted arrow indicates the putative effect of the binding of AVR9 on the HABS. (B) In Cf9 tomato plants (that do contain Cf-9), binding of AVR9 to the HABS is perceived by the Cf-9 protein. Initiation of defence responses, including a hypersensitive response (HR) might either occur via a putative cytoplasmic signalling domain of the HABS or (C) via an additional transducer protein. Note that the situation presented in figure 1B and 1C will result in resistance of the plant, whereas the situation presented in figure 1A results in (enhanced?) susceptibility of the plant towards *C. fulvum*. In each figure the cytoplasmic side of the plasma-membrane is situated at the bottom.

transmembrane protein with an extracellular LRR region (Dixon *et al.*, 1996). As *Cf-5* is over 90% identical to *Cf-2*, the encoded proteins are thought to initiate the same defence signalling pathway (Dixon *et al.*, 1998). Therefore, Rcr3 was proposed to play a role upstream of this common pathway. Recently, the *Rcr3* gene has been isolated and the predicted gene product shows homology to secreted cysteine proteases (J. Krueger, C.M. Thomas, C. Golstein, M.S. Dixon and J.D.G. Jones, personal communication). The fact that Rcr3 is required for Cf-2-mediated resistance, together with the predicted presence in the apoplastic space, suggests that it might mediate the perception of AVR2 by Cf-2. To allow dissection of the biochemical mechanism of perception of AVR2 by Cf-2, a cloning strategy for *Avr2* was initiated (Chapter 4). *Avr2* encodes a small, cysteine-rich protein with an N-terminal signal peptide for secretion. Searches in databases did not reveal homology with other genes or proteins (Chapter 4). With the cloning of *Avr2*, all three components have become available to study the biochemical mechanism that is underlying the perception of AVR2 by Cf-2. As depicted in figure 2, we propose that AVR2 binds to Rcr3 in the apoplast, whether Cf-2 is present or not. When present, Cf-2 perceives the binding of AVR2 to Rcr3, resulting in the activation of defence responses (Fig. 2B). As none of these three proteins contains an obvious cytoplasmic signalling domain, we expect that defence responses will be induced by a transducer protein. Considering the predicted protease activity of Rcr3, it might be part of a basal defence mechanism of the plant and thereby be the virulence target of AVR2. The putative virulence function of AVR2 might be inhibition of the protease activity of Rcr3. However, Rcr3 might also be involved in modification of either AVR2 or Cf-2 to allow specific recognition of the elicitor. Binding studies with AVR2, Cf-2 and/or Rcr3 should reveal whether Rcr3 indeed represents the third component that is involved in perception of AVR2 by Cf-2.

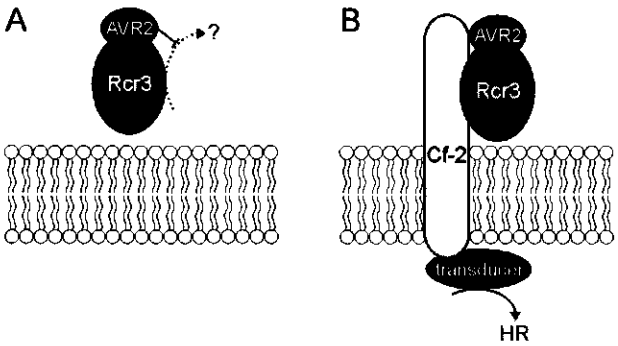

Fig. 2. Model for perception of AVR2 by tomato plants. (A) in Cf0 tomato plants (that do not contain Cf-2) AVR2 binds to Rcr3. The dotted arrow indicates the predicted protease activity of Rcr3, which might be inhibited (⊥) by AVR2. (B) In Cf2 tomato plants (that do contain Cf-2), binding of AVR2 to Rcr3 will be perceived by the Cf-2 protein. As Cf-2 itself does not contain any obvious signalling domains, initiation of defence responses, including a hypersensitive response (HR) is predicted to occur via an additional transducer protein. Note that the situation presented in figure 2B will result in resistance of the plant, whereas the situation presented in figure 2A results in (enhanced?) susceptibility of the plant towards *C. fulvum*. In each figure the cytoplasmic side of the plasma-membrane is situated at the bottom.

Elicitor perception by R proteins is predominantly indirect

For AVR9 and AVR2, experimental data are more consistent with indirect perception of the AVR protein than with a direct interaction between the *Avr* and *R* gene product (Chapter 3; Dixon *et al.*, 2000). Actually, for most gene-for-gene relationships studied so far, experimental evidence is more consistent with indirect perception of an AVR protein by an R protein than with direct interaction between these proteins (Chapter 5). Indirect perception implies that at least a third component is specifically required to mediate resistance. For several gene-for-gene relationships the nature of the putative third component is known (reviewed in Chapter 5). Their nature reveals that they all might be involved in the regulation of basal defence responses. Therefore, these putative third components are all possible virulence targets for the AVR proteins and the mechanism of perception of these AVR proteins might be consistent with the 'Guard hypothesis' (Van der Biezen and Jones, 1998). According to the 'Guard hypothesis', binding of the AVR protein to its virulence target is perceived by the matching R protein, which is 'guarding' this virulence target, resulting in resistance. In absence of the R protein, binding of the AVR protein to its virulence target will result in enhanced susceptibility of the plant, making the virulence target a key factor in the outcome of plant pathogen interactions. Whether the perception of the AVR proteins of *C. fulvum* also complies with the 'Guard hypothesis' remains to be determined.

Direct interaction between AVR and R proteins has been the main working hypothesis to explain the biochemical basis of the gene-for-gene concept for years. However, the data described in this thesis suggest that the perception of AVR proteins by R proteins is

predominantly indirect. The most important implication of this finding is that, beside the AVR and the R protein, at least a third component is specifically required to induce a defence response in most cases. This insight has important consequences for future research on gene-for-gene based resistance. Furthermore, it opens new possibilities for modern resistance breeding.

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Summary

The gene-for-gene concept postulates that for every dominant gene determining resistance in the host plant, there is a corresponding dominant gene conditioning avirulence in the pathogen. The simplest way to explain the biochemical basis of this concept is direct interaction between an elicitor protein, which is encoded by an avirulence (*Avr*) gene of the pathogen, and a receptor protein, which is encoded by the matching resistance (*R*) gene of the host. Perception of the elicitor protein by the host plant subsequently leads to the activation of defence responses, often including a hypersensitive response (HR).

The research described in this thesis is focussed on the characterisation of elicitor proteins of the fungus *Cladosporium fulvum* and the analysis of their perception by resistant tomato plants. A striking feature of all elicitor proteins of *C. fulvum* is that their mature form contains an even number of cysteine residues. These cysteine residues are thought to be involved in disulfide bridges, which are essential for proper conformation and stability of the elicitors. Mutational analysis of elicitor proteins ECP1, ECP2 and ECP5, however, revealed that the role of (the even number) of cysteine residues is more complex than anticipated, as not all cysteine residues appeared to be critical for the HR-inducing activity of the elicitor proteins (Chapter 2).

During colonisation of the apoplastic space of tomato leaves, *C. fulvum* secretes elicitor proteins into the apoplast. All *Cf* genes, mediating resistance to particular races of *C. fulvum*, are predicted to encode extracytoplasmic, membrane-anchored glycoproteins that contain many leucine-rich repeats (LRRs). LRR domains are thought to be involved in protein-protein interactions. The extracellular localisation of the LRR region of the Cf proteins is consistent with a direct, extracellular perception of the corresponding elicitor proteins. To validate this hypothesis, binding studies were performed between avirulence protein AVR9 and the matching resistance protein Cf-9. Although extensive studies were performed in a multidisciplinary collaboration to prove a direct interaction between the two proteins, no specific binding between AVR9 and Cf-9 could be detected (Chapter 3). This implies that the simplest interpretation of the gene-for-gene concept, involving direct interaction of a pathogen-derived elicitor with a matching resistance gene product, does not hold for the *Avr9/Cf-9* gene pair and that at least a third component is involved in the perception of AVR9 by Cf-9.

Also for *Avr2/Cf-2*-mediated resistance a third component, Rcr3, seems to be involved. To allow dissection of the biochemical mechanism of perception of avirulence protein AVR2, we set out to clone *Avr2* (Chapter 4). *Avr2* cDNA was cloned based on the specific HR-inducing activity of the encoded protein in Cf2 tomato plants. Like the other *Avr* genes of *C. fulvum*, *Avr2* encodes a small, secreted protein with an even number of cysteine residues. Analysis of strains of *C. fulvum* that are virulent on Cf2 tomato lines revealed various mutations in the *Avr2* ORF that all result in the production of a truncated AVR2 protein. Interestingly, an additional modification was discovered, involving the

insertion of a LINE-like element (a retrotransposable element), Cfl1, in the *Avr2* ORF. Cfl1 is the first LINE-like element identified in *C. fulvum* and provides the first example of loss of avirulence of a plant pathogen due to insertion of a retrotransposable element in an *Avr* gene. Analysis of two different rcr3-mutant Cf2 tomato plants revealed that their ability to respond to AVR2 with a HR correlates with their degree of resistance to AVR2-producing strains of *C. fulvum*. These data support a role for Rcr3 in the perception of AVR2 by Cf-2.

Direct perception of elicitor proteins by R proteins has been the prevailing working hypothesis to explain the biochemical basis of the gene-for-gene concept for years. The results of the research that is described in this thesis, however, do not support this hypothesis. Also for most other gene-for-gene relationships studied so far, experimental evidence appears to be more consistent with indirect perception of an AVR protein by an R protein (Chapter 5). Indirect perception implies that, beside the AVR and the R protein, at least a third component is required to induce defence responses. For several gene-for-gene relationships the nature of the putative third component is known. Although each of these components are suggested to be involved in basal defence mechanisms, their nature appears to be diverse. Hence, we argue that, although some elicitors might be directly perceived by the matching R protein, for most gene-for-gene relationships elicitor perception will turn out to be more complex.

Samenvatting

Volgens het gen-om-gen concept correspondeert ieder dominant gen dat resistentie geeft in de waardplant, met een dominant gen dat leidt tot avirulentie van het pathogeen. De eenvoudigste verklaring van de biochemische basis van het gen-om-gen concept bestaat uit het optreden van een directe interactie tussen een elicitoreiwit dat gecodeerd wordt door een avirulentiegen (*Avr*) van het pathogeen, en een receptoreiwit dat gecodeerd wordt door het corresponderende resistentiegen (*R*) van de waardplant. Perceptie van het elicitoreiwit door de waardplant leidt vervolgens tot het activeren van afweerreacties, zoals de overgevoeligheidsreactie (HR).

Het onderzoek dat beschreven staat in dit proefschrift is gericht op het karakteriseren van elicitoreiwitten van de pathogene schimmel *Cladosporium fulvum* en het analyseren van de manier waarop die eiwitten door resistente tomatenplanten worden herkend. Een opvallend kenmerk van alle elicitoreiwitten van *C. fulvum* is dat ze een even aantal cysteine residuen bevatten. Het vermoeden bestond dat deze cysteine residuen zwavelbruggen vormen, die essentieel zijn voor een goede conformatie en stabiliteit van de elicitoren. Mutatieanalyse van de elicitoreiwitten ECP1, ECP2 en ECP5 bracht echter aan het licht dat de rol van (het even aantal) cysteine residuen complexer is dan gedacht. Niet alle cysteine residuen bleken namelijk noodzakelijk te zijn voor de HR-inducerende activiteit van deze elicitoreiwitten (Hoofdstuk 2).

Gedurende de kolonisatie van de apoplastische ruimte van tomatenbladeren scheidt *C. fulvum* diverse elicitoreiwitten uit in de apoplast. Alle genen die resistentie geven tegen bepaalde fysio's van *C. fulvum* (*Cf* genen), coderen voor eiwitten die waarschijnlijk in het plasmamembraan van de plantencellen verankerd liggen. Hierbij ligt het grootste deel van de Cf eiwitten, die rijk zijn aan leucine-rijke repeats (LRRs), aan de buitenkant van de cel. LRRs zijn vermoedelijk betrokken bij eiwit-eiwit interacties. De extracellulaire lokalisatie van het LRR domein van de Cf eiwitten suggereert een directe, extracellulaire perceptie van de bijbehorende elicitoreiwitten. Om deze hypothese te toetsen werden bindingsstudies uitgevoerd tussen het avirulentie-eiwit AVR9 en het bijbehorende resistentie-eiwit Cf-9. Alhoewel in een multidisciplinair samenwerkingsverband zeer uitgebreide experimenten werden gedaan om een interactie tussen deze twee eiwitten aan te tonen, werd geen specifieke binding tussen AVR9 en Cf-9 gedetecteerd (Hoofdstuk 3). Dit impliceert dat de eenvoudigste verklaring van het gen-om-gen concept, namelijk een directe interactie tussen een elicitoreiwit en een corresponderend resistentie-eiwit, niet van toepassing is op de AVR9/Cf-9 combinatie en dat minstens een derde component betrokken is bij de perceptie van AVR9 door Cf-9.

Bij de perceptie van AVR2 door Cf-2 lijkt eveneens een derde component, Rcr3, betrokken te zijn. Om onderzoek naar het biochemische mechanisme van de perceptie van het avirulentie-eiwit AVR2 mogelijk te maken, werd besloten *Avr2* te kloneren (Hoofdstuk 4). *Avr2* cDNA werd gekloneerd op basis van de specifieke HR-inducerende activiteit van

het gecodeerde eiwit in Cf2 tomatenplanten. *Avr2* codeert, net als de andere avirulentiegenen van *C. fulvum*, voor een klein, gesecreteerd eiwit met een even aantal cysteine residuen. Analyse van isolaten van *C. fulvum* die virulent zijn op Cf2 tomatenplanten bracht diverse mutaties in het open leesraam (ORF) van *Avr2* aan het licht, die allemaal de productie van een onvolledige vorm van AVR2 tot gevolg hebben. Een bijzonder interessante modificatie die werd gevonden was de insertie van een zogenaamd 'LINE-like element' (een retrotransposon), Cfl1, in het *Avr2* ORF. Cfl1 is het eerste LINE-like element dat in *C. fulvum* aangetoond is en het vormt het eerste voorbeeld van het verlies van avirulentie van een pathogeen ten gevolge van de insertie van een retro-transposon in een *Avr* gen. Analyse van Cf2 tomatenlijnen, met twee verschillende mutaties in *rcr3*, liet zien dat hun vermogen om op AVR2 te reageren met een HR gecorreleerd is aan de mate waarin ze resistent zijn tegen AVR2-producerende isolaten van *C. fulvum*. Deze gegevens wijzen erop dat Rcr3 een rol speelt bij de perceptie van AVR2 door Cf-2.

Directe perceptie van elicitoreiwitten door resistentie-eiwitten is jarenlang de algemeen gehanteerde hypothese geweest om de biochemische basis van het gen-om-gen concept te verklaren. De resultaten van het onderzoek dat beschreven staat in dit proefschrift zijn echter niet in overeenstemming met deze hypothese. Ook voor de meeste andere gen-om-gen relaties die tot op heden bestudeerd zijn lijken de verkregen data meer consistent te zijn met indirecte perceptie van avirulentie-eiwitten door resistentie-eiwitten (Hoofdstuk 5). Indirecte perceptie impliceert dat naast het AVR en het R eiwit, minimaal een derde component specifiek vereist is om een afweerreactie te induceren. Voor een aantal gen-om-gen relaties is de aard van de derde component bekend. Alhoewel deze componenten stuk voor stuk betrokken zouden kunnen zijn bij basale afweermechanismen van de plant, is de aard van de eiwitten zeer divers. Derhalve concluderen we dat, ondanks het feit dat de perceptie van sommige avirulentie-eiwitten door het bijbehorende R eiwit wellicht direct is, de elicitorperceptie bij de meeste gen-om-gen relaties complexer zal blijken te zijn.

Nawoord

Promotieonderzoek is voor mij altijd het logische vervolg op mijn studie geweest. De vraag was alleen waar dat promotieonderzoek dan over zou moeten gaan. Toen de mogelijkheid zich voordeed om als OIO bij Fytopathologie aan *Cladosporium*-tomaat te gaan werken was die vraag echter snel beantwoord. Het project was heel uitdagend en stond garant voor veelzijdig onderzoek van DNA-niveau tot (de fysiologie van) de interactie tussen de organismen. Alhoewel de onderzoeksresultaten hebben geleid tot een andere invulling van het boekje dan oorspronkelijk was gedacht, is de veelzijdigheid van het onderzoek gelukkig niet in het gedrang gekomen. Ik heb tijdens mijn tijd bij Fyto enorm veel geleerd en daarvoor wil ik graag een aantal mensen bedanken.

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Curriculum vitae

Rianne Luderer werd op 4 oktober 1973 geboren in Velp (Gld.). Na in Dieren de lagere school doorlopen te hebben ging zij in Rozendaal naar het Rhedens Lyceum. In juni 1992 behaalde zij aan deze school het V.W.O. diploma. In datzelfde jaar begon zij aan haar studie biologie aan de Katholieke Universiteit Nijmegen. Binnen deze studie koos zij na anderhalf jaar de fysiologisch biochemische richting. De specialisatiefase van de studie omvatte twee hoofdvakstages van ongeveer een jaar. Bij de afdeling Moleculaire Plantenfysiologie deed zij tijdens haar eerste stage onderzoek naar de regulatie van DAHP-synthase onder begeleiding van Dr. J.C. Wind en Dr. A.F. Croes. Tijdens de tweede stage, bij de afdeling Microbiologie, werd onderzoek gedaan aan een methanogene *Archae* die vluchtige organische zwavelverbindingen kan afbreken onder begeleiding van Dr. B.P. Lomans en Dr. H.J.M. op den Camp. In maart 1997 behaalde zij *cum laude* haar doctoraal diploma. Eveneens in maart 1997 startte zij met haar promotieonderzoek bij het Laboratorium voor Fytopathologie van Wageningen Universiteit onder begeleiding van Dr. ir. M.H.A.J. Joosten en Prof. dr. ir. P.J.G.M. de Wit. De resultaten van dit door N.W.O. gefinancierde project staan beschreven in dit proefschrift.

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The *Cladosporium fulvum*-tomato interaction: elicitor proteins and their perception
Luderer, Rianne

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With references - With summary in Dutch

On the cover: *Cladosporium fulvum* growing in high density on plates.
Cover picture: Berry Geerligs, Beeldgroep WUR

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