The AVR9 elicitor peptide of the tomato pathogen *Cladosporium fulvum*:
Molecular aspects of recognition

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The AVR9 elicitor peptide of the tomato pathogen *Cladosporium fulvum*: Molecular aspects of recognition

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Stellingen

1. Het elicitor-receptor model, afgeleid van de gen-om-gen hypothese, geldt waarschijnlijk niet voor het genenpaar *Avr9-Cf-9*.
   Dit proefschrift.

2. Homologie in aminozuurvolgorde of structuur vormt onvoldoende basis voor het betrouwbaar voorspellen van de functie van een eiwit.
   Dit proefschrift.

3. Het nematode resistentiegen $Hs1^{pro-1}$ kan beter geclassificeerd worden als Leucine-Rich Protein, dan als Leucine-Rich Repeat Protein.
   Science 275, 832-834; Prog. Biophys. Mol. Biol. 65, 1-44.

4. De conclusie van Keen dat victorine een elicitor is van afweerreacties is onjuist, zelfs als het Victoria blight vatbaarheidsgen *Vb* identiek blijkt te zijn aan het *Puccinia coronata* resistentiegen *Pc-2*.
   Plant Mol. Biol. 19, 109-122.

5. De naamgeving van sommige virusresistentiegenen is niet gericht op algemeen begrip en duidelijkheid.
   Plant Cell 8, 169-178 (N' gene); Mol. Plant-Microbe Interact. 10, 709-715 (N gene); and Mol Plant-Microbe Interact. 8, 85-89 (Nucleoprotein N gene).

6. De term avirulentiefactor is misleidend en zou moeten worden vervangen door: 'Fysio-specifieke elicitor' (Race-specific elicitor). AVR9 zou derhalve beter RSE9 genoemd kunnen worden.

7. Wetenschappers die doen belangrijker vinden dan denken benutten de beschikbare mankracht en hulpmiddelen niet optimaal.

8. De promotieplechtigheid is het kostbaarste uur van het hele promotieonderzoek.

9. Dat wetenschappelijk onderzoek inzicht geeft en niet altijd problemen kan oplossen maakt dat de utiliteitsparagraaf van onderzoeksvoorstellen veelal gevuld is met 'wishful thinking'.

10. Een overeenkomst tussen de LUW en de Elfstedenvereniging is dat hun leeftijdsopbouw de kansen van aankomend talent belemmert.

11. Dat het als objectief beschouwde NOS journaal CO_2 omschrijft als 'een zeer giftige stof' geeft te denken over de feitelijke juistheid van informatievoorziening op televisie.

12. 'This is the first report about....', is meestal niet waar en overbodig.

13. Discriminatie is nooit positief.

Stellingen behorende bij het proefschrift van Miriam Kooman-Gersmann: The AVR9 elicitor peptide of the tomato pathogen *Cladosporium fulvum*: Molecular aspects of recognition.

Most impediments to scientific understanding are conceptual locks, not factual lacks.
S.J. Gould, Bully for Brontosaurus, p 256.

VOORWOORD

Alhoewel de titel 'voorwoord' het niet doet vermoeden, is dit het laatste onderdeel van dit proefschrift dat ik schrijf. Dit doet geen recht aan het belang van dit voorwoord, aangezien dit proefschrift nooit tot stand zou zijn gekomen zonder de hulp, inzet, ondersteuning en begeleiding van velen. Alhoewel hier alleen de meest direct betrokkenen met name wordt genoemd, wil ik hierbij ook niet genoemde vrienden en familie bedanken voor hun interesse en ondersteuning.

De afgelopen 4 jaar heb ik met veel plezier gewerkt aan een klein eiwitje, 'AVR9'. Klein is niet hetzelfde als onbelangrijk (ik kan het weten) en de afgelopen jaren hebben een groot aantal onderzoekers zich direct of indirect beziggehouden met het AVR9 onderzoek. Zij hebben allen in meer of mindere mate bijgedragen aan het tot stand komen van dit proefschrift. Ten eerste bedank ik natuurlijk de 'koning' van AVR9, Pierre. Pierre, jouw enthousiasme en geweldige stroom ideeën werkten vaak aanstekelijk en hebben er mede toe geleid dat ik me in de afgelopen jaren geen moment heb verveeld en verder op een heel prettige, zelfstandige manier heb kunnen werken. Jammer dat een dag maar 24 uur heeft. Dan 'onderkoning' Guy, lange tijd officieuze, altijd echte, en nu toch officiële co-promoter. Je was altijd bereid was om de knopen uit m'n planning, gedachten en vooral uit mijn artikelen te ontwarren. Veel dank daarvoor. Een aantal van de proeven die in dit proefschrift staan beschreven zijn uitgevoerd door Rob of door Paul. Zonder jullie stond ik nu nog op het lab bij fyto, waar het zonder jullie overigens ook een stuk minder gezellig zou zijn geweest. Dan waren er de afgelopen jaren vele tijdelijke AVR9-onderzoekers en -medewerkers. Ralph, Ton, Jos, Juul, Franck en vele anderen zorgden voor leuke discussies en gezellige momenten. Verder bedank ik de studenten, Erwin, Patrick, André, Bianca, Miriam en Marianne. Alhoewel jullie werk helaas niet evenredig vertegenwoordigd is in dit boekje, heb ik van jullie allemaal veel geleerd en geprofiteerd van het door jullie verrichtte onderzoek.

Hoewel het bij het lezen van dit proefschrift nauwelijks opvalt, is AVR9 niet het enige eiwit dat door *Cladosporium fulvum* geproduceerd wordt. Van de 'niet-AVR9' Clado's was Matthieu altijd de 'rots in de branding van het schimmelwerk'. Bedankt voor liters IV, vele discussies en het doorlezen van artikelen. Naast de Clado-groep hebben ook de andere collega's op fyto bijgedragen aan de prettige werksfeer. Discussies in het "AIO-clubje" hebben mij erg gestimuleerd en ook zeker bijgedragen aan het

verbeteren van de kwaliteit van dit proefschrift. Ik hoop dat de 'club' nog lang mag blijven bestaan. Verder waren Elly, Ali, Baukje, Ria en Willem altijd bereid tot ondersteuning bij lastige bestellingen of als ik voor de zoveelste keer mijn kopieerkaart kwijt was. De medewerkers van de kassen van het Binnenhavencomplex, met name Bertus en Bert, dank ik voor de goede zorgen voor 'mijn' plantjes en voor hun altijd flexibele opstelling als ik weer eens onverwacht planten kwam stelen voor niet-geplande proeven. Ook de medewerkers van de 'Technische Dienst', vooral Willy en Gerard, stonden altijd klaar voor het maken buizen of repareren van apparatuur. Bij Duotone heeft vooral Wim gezorgd voor mooie en duidelijke foto's en dia's. Met behulp van deze foto's, maar vooral door het werk van Cathy, is de omslag veel mooier geworden dan verwacht.

Buiten de vakgroep hebben vele *Cladosporium* geïnteresseerden bijgedragen met ideeën, begeleiding, experimenten en door het beschikbaar stellen van onderzoeksmateriaal. Dank aan Jacques Vervoort en Henno van den Hooven voor 'structurele' discussie en aan onderzoekers en projectleiders van het STW-project '*Cf4*, of was het nou *Cf-9*?'. I would also like to thank the people from the HC&M programme 'Signal perception and transduction in plant-fungus interactions' and the 'Norwich-group' for helpful discussions. Thorsten Nürnberger and Dirk Nennstiel tought me how to do binding studies and Paul van den Hoeven showed me the 'ins and outs' of membrane isolations. Both skills have proven essential for the research described in this thesis.

Twee mensen die in de afgelopen jaren altijd klaarstonden als 'uitlaatklep' en (nu nog) steun geven, zijn mijn paranimfen, Richard en Jeanine. Van dichtbij en op afstand zorgden jullie voor de nodige stimulans om dit boekje af te maken. Richard, I have greatly appreciated the discussions we have had and I hope we will continue them outside the lab in future. Jeanine, op afstand, maar door E-mail toch niet echt ver, las je m'n belevenissen en gemopper. Met enige vertraging nu toch paranimf. Mijn ouders, maar vooral mijn vader, bedank ik voor de enorme belangstelling tijdens dit promotieonderzoek en ongedwongen stimulans bij het maken van belangrijke keuzen. Tot slot de belangrijkste. Peter, zonder jouw relativeringsvermogen en je heldere en analytische kijk op mijn werk en artikelen was dit proefschrift minder efficiënt tot stand gekomen. Jouw input varieerde al naar gelang mijn vraag en was zowel van veraf (Amerika) als dichtbij heerlijk.
Nu is er weer tijd.

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CHAPTER 1

CHARACTERIZATION OF ELICITOR BINDING SITES; AN OVERVIEW

Initiation of plant defense

Plants have developed a diverse array of defense mechanisms to protect themselves against invading pathogens. The initial, and crucial event to activate the plant defense is detection or 'recognition' of the invading pathogen. Recognition is based on the perception of chemical signals that are produced during infection of the plant by the pathogen. Chemical substances that induce plant defense reactions, are collectively known as elicitors, and have first been described in the seventies (Keen et al., 1972; Anderson-Prouty and Albersheim, 1975; Keen and Brügger, 1977). New techniques, developed during the last two decades, have significantly increased our understanding of plant-pathogen interactions at the molecular level and have generated further insight in how plants can recognize elicitors. A vast number of fungal, bacterial and viral substances were shown to have elicitor activity, including carbohydrates, glycoproteins, peptides and fatty acids (reviewed by Kogel and Beißmann, 1991). Elicitors are thought to be recognized by plant cells through receptors, which are often located on the plasma membrane (Hahn, 1996a and b). Binding of an elicitor to its receptor represents the first step in the induction of a cascade of defense responses. The signals of this cascade will activate genes involved in plant defense reactions. Pathogen-derived elicitors can be roughly divided into two groups: (1) non race-specific elicitors, which are recognized at the plant species, plant family, or plant kingdom level and (2) race-specific elicitors, which are specifically recognized at the sub-species level. In this chapter, in addition to the above mentioned elicitors, fungal toxins will be discussed, since these were among the first fungal signals for which the interaction with plants has been studied at the molecular level.

Elicitor binding sites

In 1993, at the start of the research project described in this thesis, binding sites of plants had been identified for only a limited number of fungal compounds. These included the toxins fusicoccin (Dohrmann et al., 1977), victorin (Wolpert and Macko, 1989), and T-toxins (Levings and Siedow, 1992), and the non race-specific elicitors ß-glucan (Schmidt and Ebel, 1987), the *Puccinia graminis* f. sp. *tritici* glycopeptide (Pgt; Kogel et al., 1991), and the *Phytophthora megasperma* glycoprotein (Pmg; Renelt et al., 1993). More recently, additional binding proteins have been characterized for fungal toxins and non race-specific elicitors and the first results on isolation of plant binding sites have been reported. An overview of all bacterial and fungal components for which binding sites have been characterized in plants is given in Table 1.1. In the next paragraphs, the progress that has been made over the last two decades in our knowledge on elicitor recognition is discussed, with emphasis on binding sites for fungal toxins, non race-specific elicitors and race-specific elicitors.

Binding sites for fungal toxins

Although not all fungal toxins are by definition elicitors of plant defense, toxins do initiate a response in plant cells and early work on high-affinity receptors for pathogen-induced signals focused mainly on toxins. For host-selective toxins, there is specificity at the plant species or genotype level and the toxin determines the host range of the pathogen. For most toxic compounds, however, there is limited information on their role in determining the outcome of an interaction (Walton, 1996). In general, toxins are agents of compatibility (successful infection), whereas elicitors are agents of incompatibility (unsuccessful infection, initiation of active resistance response). Only those fungal toxins are discussed, for which binding sites have been described.

The first plant binding site for a fungal component was reported as early as 1977, when the non host-selective fungal toxin fusicoccin (FC) from *Fusicoccum amygdali,* a pathogen of almond and peach, was found to bind to plasma membranes of corn (Dohrmann et al., 1977). Since then, the FC binding protein has been the most extensively studied binding protein in plants. It has both high- and low-affinity for FC (Basel et al., 1994) and is present on plasma membranes of all higher plants tested (Marrè, 1979). Several authors reported the purification

Table 1.1 Fungal and bacterial compounds for which binding sites have been identified in plants

Compound	Type	Pathogen	Plant species	Binding characteristics (localization of binding site; K_d)	Reference(s)
Fusicoccin	Toxin	Fusicoccum amygdali Del.	All higher plants	Plasma membrane; ~ 0.7 nM & ~20 nM	Dohrmann et al., 1977, Marrè, 1979, many others
Victorin	Toxin	*Cochliobolus carbonum*	Oats	Mitochondria; K_d not published.	Wolpert and Macko, 1989
T-toxins	Toxins	*Phyllosticta maydis/Bipolaris maydis*	Maize	Mitochondria; ~ 50 nM	Siedow et al., 1995
Lipo-poly-saccharide	Toxin	*Verticillium dahliae*	Cotton	Plasma membrane; ~14 nM	Meyer and Dubery, 1993; Dubery and Meyer, 1996
β-glucans	Non Race-sp. Elicitor	*Phytophthora megasperma*	Fabaceae	Plasma membrane; ~1 nM	Schmidt and Ebel, 1987; Cosio et al., 1996
Pmg[1] elicitor	Non Race-sp. Elicitor	*Phytophthora megasperma*	Parsley	Plasma membrane; ~2.4 nM	Renelt et al., 1993; Nürnberger et al., 1994
Pgt[2] glyco-protein	Non Race-sp. Elicitor	*Puccinia graminis*	Barley & Wheat	Plasma membrane; ~2000 nM	Kogel et al., 1991
Invertase fragments (gp 8c)	Non Race-sp. Elicitor	yeast	Tomato	Plasma membrane; ~5 nM	Basse et al., 1993
Chitin fragments	Non Race-sp. Elicitor	fungal cell walls	Rice & Tomato	Membrane; ~5 nM (Rice) & ~23 nM (Tomato)	Shibuya et al., 1993 (Rice); Baureithel et al., 1994 (Tomato).
NodRm	Nod factor	*Rhizobium meliloti*	*Medicago* spp.	Membrane; ~ 1.9 nM	Niebel et al., 1997
EIX[3]	Non Race-sp. Elicitor	*Trichoderma viride*	Tobacco & Tomato	Plasma membrane; ~ 6.2 nM	Hanania and Avni, 1997
Cryptogein	Non Race-sp. Elicitor	*Phytophthora* & *Pythium* spp.	Tobacco	Plasma membrane; ~2 nM	Wendehenne et al., 1995
AVR9	Race-sp. Elicitor	*Cladosporium fulvum*	Tomato	Plasma membrane; ~0.07 nM	This thesis
Syringolides	Race-sp. Elicitors	*Pseudomonas syringae*	Soybean	Soluble fraction; ~8.7 nM	Ji et al., 1997

[1] *Phytophthora megasperma* glycoprotein

[2] *Puccinia graminis* f. sp. *tritici*

[3] Ethylene-inducing xylanase

and characterization of FC binding proteins from different plant species (amongst others Pesci et al., 1979: maize; de Boer et al., 1989: oat). Only recently, the FC binding protein has been sequenced and the protein was found to belong to the so-called 14-3-3 protein family (Marra et al., 1994; Korthout and de Boer, 1994). This relationship has not given much insight in the function of the binding protein, since the 14-3-3 proteins are involved in the regulation of a wide range of signals, varying from growth and development in mammals, to light harvesting in plants (reviewed by Aitken, 1992).

Two other fungal toxins for which binding sites in plants have been reported are victorin and T-toxins. Surprisingly, the reported binding sites are not located on the plasma membrane. The binding site for the host-selective toxin victorin, a cyclic pentapeptide from *Cochliobolus victoriae* is localized in the mitochondria (Wolpert and Macko, 1989). Victorin binds to two different components of the mitochondrial multi-enzyme glycine decarboxylase complex, a 15 kDa, and a 100 kDa protein (Navarra and Wolpert, 1995). These binding sites are present both in toxin-sensitive and toxin-resistant oats genotypes. The mechanism of host-selectivity of the toxin is not yet understood, but deduced from the different *in vivo* and *in vitro* binding data, resistance is proposed to result from the inability of mitochondria of resistant plants to take up the toxin. Thus, host-selectivity is not directly determined by the binding process. The T-toxins comprise a set of toxins produced by the fungal pathogens *Phyllosticta maydis* and *Bipolaris maydis* (Levings and Siedow, 1992). T-toxins form another example of toxins for which a mitochondrial binding site has been reported. Maize with T-cytoplasm (*cms-T*), carries a cytoplasmically inherited male sterility factor and is sensitive to T-toxins. An unusual mitochondrial gene, T-*urf13*, is responsible for sensitivity to the toxins. The encoded protein, URF13, is an integral mitochondrial membrane protein, with three membrane spanning domains. Binding studies with [^{3}H]-labeled toxins showed no significant difference in binding to mitochondrial membranes of normal or *cms-T* maize (Franzen et al., 1987). However, more recent studies have shown that expression of T-*urf13* in *E. coli* results in specific toxin binding (Braun et al., 1990). Thus, URF13 binds T-toxins and determines host-selectivity.

Binding studies have also been performed with a phytotoxic protein-lipopolysaccharide (PLP) complex from *Verticillium dahliae.* This complex is produced by virulent strains of *Verticillium* and can induce wilt disease symptoms. The presence of a high-affinity binding site for the PLP complex on plasma

membranes of cotton has been demonstrated by Meyer and Dubery (1993). This binding site was also shown to be present on protoplasts, where the binding affinity was nearly identical to that of purified plasma membranes. No significant difference in binding affinity was found between cultivars exhibiting resistance or susceptibility towards the pathogen, but the number of binding sites in resistant cultivars was significantly higher (Dubery and Meyer, 1996). It was suggested that the increased binding capacity of toxin-resistant plants reflects a role in the induction of defense responses. Alternatively, the toxin may inhibit the activity of an essential plant protein, and toxin susceptibility is overcome by increasing the expression of that protein.

Although fungal toxins were among the first fungal components for which binding studies were performed, only recently, more insight in the nature of the binding proteins was obtained by the isolation of both fusicoccin and victorin binding proteins and the characterization of URF13. It seems that the toxin binding proteins are often related to the toxic activity of their ligands, and are not necessarily involved in recognition of a fungus by the plant. Resistance against a toxin can depend on the presence or the expression level of the binding protein (T-toxins and PLP-complex, respectively), on the uptake or secretion of the toxin (the possible mechanism for host-selectivity of victorin) or on the presence of detoxifying enzymes (for instance HC-toxin, which is not discussed here, since no binding studies have been published for HC-toxin). For fusicoccin no 'toxin-resistant' plants have been described and toxic effects are initiated in all plant species tested.

Binding sites for non race-specific elicitors

Binding proteins for elicitors are proposed to be directly involved in recognition of the pathogen by the plant. Binding sites for elicitors were first described for the fungal ß-glucans, isolated from *Phytophthora megasperma* (recently renamed *P. sojae*). ß-Glucans have the ability to induce phytoalexin accumulation in soybean tissue and a binding site for hepta-ß-glucoside was shown to be present in membranes of soybean (Schmidt and Ebel, 1987; Cosio et al., 1988). The ability of ß-glucans to bind to soybean membranes corresponds to their ability to induce phytoalexin accumulation. More recently it has been shown that not only in soybean, but also in other species of the family Fabaceae, ß-glucans can induce phytoalexin accumulation and bind to membranes (Cosio et a., 1996). A ß-glucan binding protein has been isolated from soybean, which has no homology to proteins

of known function, nor shows typical features of signaling domains or receptor binding sites (Umemoto et al., 1996).

Other fungal oligosaccharins for which binding sites have been identified in plants are chitin fragments. Chitin is a major constituent of cell walls of fungi. Oligosaccharide fragments of chitin can induce various defense responses in plant cells (Baureithel et al., 1994). Binding sites for chitin fragments have been described by various groups. Shibuya et al. (1993) identified a binding site for N-acetylchitooctaose in membrane fractions from rice cell cultures. Tomato cells also have chitin binding sites, as shown for a variety of chitin fragments with different degrees of polymerization (Baureithel et al., 1994). The biological activity of the chitin fragments corresponds to their binding affinity, indicating that the binding site plays a role in the initiation of defense responses. Structurally related to the chitin fragments are the Nod factors from *Rhizobiaceae,* lipochitooligosaccharides with an *N*-linked fatty acid. Nod factors elicit a number of developmental changes in roots of leguminous plants. NodRm from *Rhizobium meliloti* elicits the responses that eventually lead to the formation of nodules in *Medicago* spp. Binding sites for NodRm are present in root fractions of both leguminous and non-leguminous plants (Bono et al., 1995). Recently, two classes of binding sites for NodRm were reported in *Medicago* cell suspensions and both were localized in the microsomal membrane fraction (Niebel et al., 1997). Whether the binding sites are important for symbiosis remains to be determined.

Binding studies were also performed for fungal glycoproteins. Kogel et al. (1991) reported binding of a glycoprotein elicitor (Pgt elicitor) from germ-tube walls of *Puccinia graminis* f. sp. *tritici.* The elicitor induces lignification in a cultivar non-specific manner in wheat and barley. Coinciding with this lignification, a binding site for the Pgt elicitor is present on wheat and barley plasma membranes. Non—'natural' fungal glycopeptides, such as the glycopeptides that are generated by enzymatic cleavage of yeast invertase also initiate a variety of responses in tomato cell suspension cultures These include ethylene formation and the production of phenyl ammonia lyase. A tomato binding site has been identified for one of these peptides (gp 8c; Basse et al., 1993). The carbohydrate moiety of the peptide determines both the specificity of binding and the subsequent responses. Glycans, released from the glycopeptide, act as antagonists of the binding site and suppress gp 8c activity. Binding of the 42 kDa Pmg elicitor from *Phytophthora megasperma* to microsomal membranes from cultured parsley cells was initially reported by

Renelt et al. (1993). The elicitor induces a wide range of defense responses, including ion fluxes, an oxidative burst, defense-related gene activation and phytoalexin formation. A 13—amino acid peptide (Pep-13) derived from this elicitor was shown to be sufficient to specifically induce defense responses (Nürnberger et al., 1994). The specificity of mutant Pep-13 elicitors for the induction of defense responses, was directly correlated with their binding specificity, suggesting a role for the binding site in recognition and the initiation of defense. Pep-13 could be crosslinked to the high affinity binding site, a 91 kDa protein (Nürnberger et al., 1995) and biochemical isolation of the Pep-13 binding site is in progress (D. Nennstiel, personal communication).

In addition to the binding proteins described for oligosaccharins and glycopeptides, binding proteins have also been described for peptides. These include the binding protein for the previously described Pmg elicitor. The peptide moiety rather than the glycan part of this elicitor determines its binding capacity. Furthermore, a binding protein for an elicitin has been characterized. Elicitins comprise a family of small, 10 kDa extracellular elicitor proteins that are produced by several species of *Phytophthora* and *Pythium*. They have been suggested to act as avirulence factors, since isolates of *Phytophthora parasitica* that are pathogenic on tobacco, do not produce elicitins, whereas non-pathogenic isolates do. Furthermore, elicitins induce systemic acquired resistance and leaf necrosis in tobacco plants, which mimics a hypersensitive reaction. Since no gene-for-gene interaction has been identified, and recognition occurs at the species level, they are considered non race-specific elicitors. Cryptogein, a basic elicitin produced by *Phytophthora cryptogea,* induces the production of ethylene and phytoalexins in tobacco cells and increases the pH of the culture medium. Binding sites for cryptogein have been identified in tobacco plasma membranes by Wendehenne et al. (1995). Recent data indicate that these binding sites, do not only bind cryptogein, but also bind other elicitins with equal affinity. (A. Pugin, personal communication). The role of the elicitin binding site in the induction of plant defense remains undetermined. Another fungal protein elicitor for which binding studies have been performed is a 22 kDa β-1,4-endoxylanase from *Trichoderma viride,* referred to as ethylene-inducing xylanase (EIX) (Hanania and Avni, 1997). EIX induces ethylene in different solanaceous plant species like tobacco, tomato and pepper, and responsiveness to EIX is controlled by a single dominant gene (Bailey et al., 1993). Binding of EIX correlates with the genetic ability of plants to respond to EIX, suggesting that the gene that controls the response to EIX encodes its receptor.

Apart from the ß-glucan binding site, and the binding site for the Pgt elicitor, all described binding sites for non race-specific elicitors have been characterized in the last five years. The role of the binding sites in recognition of the invading pathogen remains to be determined, but correlation has been observed between biological activity and binding affinity for several of the elicitors (β-glucans, chitins, Pmg). This suggests a role for the binding sites in the initiation of the corresponding biological response. The only elicitor binding site that has been isolated and sequenced so far is that of the non race-specific β-glucans (Umemoto et al., 1996).However, this has not provided any insight into its physiological function. It is expected that the major efforts that are now undertaken to isolate binding sites of the non race-specific elicitors and to clone their encoding genes will lead to a better understanding of the function of elicitor receptors. Whether the binding proteins for non race-specific elicitors are directly or indirectly related to the physiological function of the elicitors, will then become clear.

Recognition of race-specific elicitors

The race-specific elicitors form a group of elicitors, for which recognition is governed by the gene-for-gene model, first described by Flor (1942). This model states that active resistance in plants is based on the presence of a resistance gene in the plant and a corresponding avirulence gene encoding the race-specific elicitor in the fungus. Since its first description many plant—pathogen interactions were found to be governed by gene-for-gene relationships, including interactions between plants and viruses, bacteria, fungi, nematodes and even insects (De Wit, 1995). Gene-for-gene—based resistance is generally characterized by the hypersensitive response (HR), the localized death of a few host cells surrounding the invading pathogen, which is accompanied by additional defense responses. HR restricts the invading pathogen to the infection site, possibly by depriving the pathogen from nutrients, or by releasing antimicrobial compounds from the dying cells. It has been proposed that HR is triggered by a direct interaction between the product of an avirulence gene, the race-specific elicitor, and the product of its corresponding plant resistance gene (Gabriel and Rolfe, 1990).

Many genes involved in recognition of race-specific elicitors have been mapped, and several avirulence and resistance genes have now been cloned. Avirulence genes were first defined by genetic crosses between different virulent and avirulent races of the fungal pathogen *Melampsora lini,* which were tested on matching susceptible and resistant flax plants (Flor, 1942). For many plant-fungus

interactions, detailed information is available on the loci involved in avirulence and resistance. Well-defined interactions include amongst others the *Bremia lactuca*-lettuce interaction (Ilott et al., 1989), and the *Melampsora lini*-flax interaction (Timmis et al., 1990). Although the loci of the dominant avirulence factors have been analyzed for many fungi, the first avirulence genes have been cloned from bacteria. Over 30 bacterial avirulence genes have been cloned and characterized. For most of these genes, their deduced amino acid sequences do not give insight in their function, or the possible mechanism by which they are recognized (Dangl, 1995). Positive functions in virulence have been described for only a very limited number of bacterial avirulence factors (Dangl, 1995). Only for the AvrD elicitor of *P. syringae*, a signal molecule has been identified. The *AvrD* gene probably encodes an enzyme, and the product of this enzyme are elicitor-active syringolides (Dangl, 1994; Ji et al., 1997). Recent studies have given some clues why infiltration of purified bacterial AVR products into the apoplast of host plants did not induce an HR. Evidence accumulates that bacterial avirulence factors can be delivered directly into the host cell by the type III secretion pathway, encoded by the *Hrp* (hypersensitive response and pathogenicity) gene cluster (reviewed by Bonas and Van den Ackerveken, 1997). Type III secretion does not require a classical N-terminal signal peptide on the secreted proteins and bacterial *avr* genes encode hydrophilic proteins that lack such a signal sequence. It has been proposed that recognition of these bacterial elicitors also takes place inside the plant cell (Leister et al., 1996). Intracellular recognition of avirulence factors has been studied by transient or stable expression of these factors inside the cell. Elicitors for which intracellular recognition has been shown include AvrBs3 (Van den Ackerveken, 1996), AvrRpt2 (Leister et al., 1996), AvrRpm1/AvrB (Gopalan et al., 1996), and AvrPto (Tang et al., 1996; Scofield et al., 1996), of which the latter three are included in Table 1.2. For plant viruses, genes with known functions, like the genes encoding movement protein, replicase, and coat protein have been assigned as determinants of avirulence (Meshi et al., 1989; Padgett et al., 1997; Taraporewala and Culver, 1996). The specificity of viral avirulence genes is often determined by a limited number of amino acids. The proposed intracellular recognition of bacterial and viral avirulence factors coincides with intracellular location of the corresponding resistance genes, with the exception of *Xa21*. Only for the bacterial avirulence factor AvrPto, evidence of direct physical interaction between the resistance gene product (Pto) and the avirulence factor has been reported (Tang et al., 1996; Scofield et al., 1996).

At the start of the research project described in this thesis, in 1993, the only cloned fungal avirulence gene was the *Avr*9 gene from *Cladosporium fulvum* (Van Kan et al., 1991). More recently, the *Avr*4 gene from *C. fulvum* has also been cloned (Joosten et al., 1994). These genes will be discussed in some detail in the paragraph dealing with the *C. fulvum*-tomato interaction (1.3). Except for these two mentioned avirulence-genes, very limited information is available on other fungal avirulence genes. The *Nip1* gene from *Rhynchosporium secalis* has been cloned (Rohe et al., 1995). Strains carrying *Nip1* are avirulent on barley plants that carry the *Rrs1* resistance gene, which has been shown by transformation of a virulent race with the *Nip1* gene, which renders the race avirulent. Also the *AVR2-YAMO* avirulence gene from *Magnaporthe grisea,* has been cloned, which determines avirulence on rice cultivar Yashiro-mochi (in Valent and Chumley, 1994).

Notwithstanding the fact that several bacterial, viral and fungal avirulence genes have been cloned, the characterization of binding sites for race-specific elicitors is less well advanced than the characterization of binding sites for toxins and non race-specific elicitors. The three reported fungal avirulence genes (*Avr*9, *Avr*4 and *Nip1*), all encode small extracellular cysteine-rich peptides. These extracellular fungal elicitors are thought to be recognized by their extracellular resistance gene products, encoding the elicitor receptors. Isolation of their receptor could confirm the elicitor-receptor model by showing that the receptor is encoded by the corresponding resistance gene product. Except for the binding site for the AVR9 elicitor peptide of *C. fulvum*, which is described in this thesis, and the recent report on the binding site for syringolides in soybean (Ji et al., 1997), no binding sites for race-specific elicitors have been identified biochemically.

In 1993, also no plant resistance genes had yet been identified, although many resistance genes were already mapped. In this study, interaction between AVR9 and the protein encoded by its corresponding resistance gene (*Cf-9*) was proposed and put forward as one of the possibilities to isolate this gene. The two other major strategies that have been utilized to isolate resistance genes were map-based cloning and transposon tagging. The latter two strategies have proven successful, resulting in the cloning of almost 20 resistance genes (Table 1.2), and the flow seems incessant. For only a few resistance genes, their corresponding avirulence gene has been cloned as well (Table 1.2). The structural features of resistance genes and their encoded products can give insight in recognition of elicitors by plants. Most resistance genes exist as multigene families, suggesting an

evolutionary mechanism, in which new resistance specificities arise by duplication of an ancestral resistance gene, followed by diversion of sequences. A distinctive feature of most of the resistance genes are the leucine-rich repeat motifs (LRRs), which are either located intracellularly, or extracellularly. LRR motifs are known to be involved in protein-protein interactions (Kobe and Deisenhofer, 1995). Extensive reviews of the LRR family of proteins have appeared, describing their structure and possible functions in recognition or signaling (Kobe and Deisenhofer, 1995; Buchanan and Gay, 1996; Jones and Jones, 1996). As mentioned before, the predicted location of the resistance gene products coincides with the predicted location of the corresponding elicitors, which suggests that resistance gene products are involved in initial steps of elicitor recognition. Other features of resistance gene products include kinase domains, suggesting a role in signal transduction cascades, and nucleotide binding motifs, implying binding of ATP or GTP to activate kinases. Many review articles have appeared recently, describing the different classes of resistance genes and their possible evolution and function. (Staskawicz et al., 1995; Jones and Jones, 1996; Hammond-Kosack, et al., 1996; Hammond-Kosack and Jones, 1997; de Wit, 1997; Parker and Coleman, 1997). An overview of the cloned genes and their general features is shown in Table 1.2.

Table 1.2 Cloned resistance genes and their (potentially) matching avirulence factors.

Cloned Resistance gene	Isolated from	features[1]	Matching avirulence gene[2]	Pathogen, which is Bacterial (B), Fungal (F), Viral (V) or Nematode (N)	Reference(s)
Cf-2	Tomato	LRR,TM,SP	*Avr2*	*Cladosporium fulvum* (F)	Dixon et al., 1996
Cf-4	Tomato	LRR,TM,SP	*Avr4* (C)	*Cladosporium fulvum* (F)	In: Jones and Jones, 1996
Cf-4A (partial resistance)	Tomato	LRR,TM,SP	not known	*Cladosporium fulvum* (F)	Takken et al., 1997
Cf-5	Tomato	LRR,TM,SP	*Avr5*	*Cladosporium fulvum* (F)	In: Jones et al., 1996
Cf-9	Tomato	LRR,TM,SP	*Avr9* (C)	*Cladosporium fulvum* (F)	Jones et al., 1994
Hs1^{pro-1}	Sugar Beet	LRR,TM,SP	not known	*Heterodera schachtii Schmidt* (N)	Cai et al., 1997
I2C (partial resistance)	Tomato	LRR,NBS	not known (race 2)	*Fusarium oxysporum* (F)	Ori et al., 1997
I2	Tomato	LRR,NBS	not known (race 2)	*Fusarium oxysporum* (F)	Simons et al., 1997a
L6	Flax	LRR,NBS, (SP)	*A-L6*	*Melampsora lini* (F)	Lawrence et al., 1995
M	Flax	LRR,NBS	*A-M*	*Melampsora lini* (F)	Anderson et al., 1997
N	Tobacco	LRR,NBS	replicase (C)	Tobacco mosaic virus (V)	Whitham et al., 1994; Padgett et al., 1997
RPP5	Arabidopsis	LRR,NBS	not known	*Peronospora parasitica* (F)	Parker et al., 1997
Pto	Tomato	PK	*AvrPto*	*Pseudomonas syringae* pv *tomato* (B)	Martin et al., 1993
Prf	Tomato	LRR,NBS,LZ	*AvrPto*	*Pseudomonas syringae* pv *tomato* (B)	Salmeron et al., 1996
RPM1/RPS3	Arabidopsis	LRR,NBS,LZ	*AvrRpm1/ AvrB*	*Pseudomonas syringae* pv *tomato* (B)	Grant et al., 1995/ Bisgrove et al., 1994
RPS2	Arabidopsis	LRR,(TM), NBS,LZ	*AvrRpt2 (AvrB)*	*Pseudomonas syringae* pv *phaseolicola* (B)	Bent et al., 1994
Xa21	Rice	SP,LRR,TM, PK	not known	*Xanthomonas oryzae* pv. oryzae (B)	Song et al., 1995; Wang et al., 1996
Hm1	Maize	O: Toxin reductase	detoxifies HC toxin	*Cochliobolus carbonum* (F)	Johall and Briggs., 1992
Mlo	Barley	O: Membrane span. Helices	none	*Erysiphe graminis* f. sp. *Hordei* (F)	Büschges., 1997; Simons et al., 1997b

[1] The general features of the resistance genes include Signal peptide (SP), Transmembrane region (TM), Protein kinase (PK), Leucine-rich repeats (LRRs), Nucleotide binding site (NBS), Leucine zipper (LZ), and Other features (O). The genes are ordered by their general features and placed in alphabetical order within each class.

[2] (C) Indicates that the avirulence gene has been cloned.

Cladosporium fulvum-*tomato as a model system to study gene-for-gene interactions*

The interaction between the fungal pathogen *Cladosporium fulvum* and tomato complies with the gene-for-gene relationship. The interaction has been studied extensively over the last decade (reviewed by Van den Ackerveken and de Wit, 1994; Joosten et al., 1997a). *C. fulvum* is a specialized, biotrophic pathogen, which can only infect tomato and causes leaf mold. Under humid conditions conidia of *C. fulvum* will germinate and form runner hyphae on the lower side of the leaf. These will grow randomly over the leaf surface, until they encounter and subsequently enter open stomata. If no resistance genes of the plant match any of the avirulence genes of the fungus, infection will proceed and the interaction is compatible. However, when both a resistance gene and its matching avirulence gene are present, the plant recognizes the fungus and will initiate an active defense response, the so-called hypersensitive response (HR), which can effectively stop fungal growth. Many genes for resistance to *C. fulvum* have been transferred from wild species to cultivated tomato. Six of these are available in near-isogenic lines of the tomato cultivar Moneymaker: MM-Cf1, MM-Cf2, MM-Cf3, MM-Cf4, MM-Cf5, and MM-Cf9, carrying resistance gene *Cf-1, Cf-2, Cf-3, Cf-4, Cf-5*, and *Cf-9* respectively. Furthermore, different physiologic races of *C. fulvum* are available, which can overcome the various resistance genes. Early studies on the *C. fulvum*-tomato interaction were focused on the isolation of elicitors of the fungus. Several non race-specific elicitor glycoproteins were isolated from *in vitro*—grown cultures of *C. fulvum* (De Wit and Rooseboom, 1980; De Wit and Kodde, 1981). In 1982, the first race-specific elicitors were found in intercellular washing fluids from compatible interactions of *C. fulvum* and tomato (De Wit and Spikman, 1982). Injection of these fluids in tomato plants resistant to the *C. fulvum* strain used, resulted in specific necrosis at the site of injection. The race-specific elicitor that induces necrosis on tomato genotypes carrying the *Cf-9* resistance gene (MM-Cf9) was isolated and purified (De Wit et al., 1985; Scholtens-Toma and de Wit, 1988) and was designated AVR9. The corresponding cDNA was isolated using degenerate oligonucleotide probes deduced from the amino acid sequence of the peptide. The *Avr*9 gene, encoding the AVR9 elicitor, specifically determines incompatibility on tomato carrying the *Cf-9* resistance gene. This was shown by transferring the *Avr*9 gene to *C. fulvum* strains that lack this gene, which resulted in avirulence on MM-Cf9 tomato. Disruption of the *Avr*9 gene from races avirulent on tomato genotypes carrying *Cf-9*, rendered the races virulent. The *Avr*9 gene encodes a 63—amino

acid pre-proprotein containing one potential glycosylation site (NSS) (Van den Ackerveken et al., 1993). Different forms of the AVR9 elicitor have been found. The mature AVR9 elicitor, predominantly present in *C. fulvum*-infected tomato plants, contains 28 amino acids (Van den Ackerveken et al., 1993). A variety of AVR9 peptides, varying from 28 to 34 amino acids have been identified in culture filtrates of *in vitro* grown transgenic *C. fulvum* overexpressing the AVR9 elicitor (Van den Ackerveken et al., 1993; De Wit, unpublished). The possibility to isolate large quantities of the AVR9 elicitor, enabled the determination of the global structure of the AVR9 peptide by two-dimensional ^{1}H-NMR (Vervoort et al., 1997). The AVR9 elicitor has 3 antiparallel β-sheets and 3 disulfide bonds that are arranged in a cystine knot (Isaacs, 1995). The AVR4 elicitor was isolated following a similar strategy as used for the isolation of AVR9. By using degenerate primers, the *Avr*4 gene has been cloned. As opposed to *Avr*9, races of *C. fulvum* which are virulent on MM-Cf4 tomato plants, do not lack the *Avr*4 gene, but show point mutations in this gene, leading to unstable gene products (Joosten et al., 1994; Joosten et al., 1997b).The mature AVR4 peptide elicitor, of which no structure-activity data are yet available, consists of 86 amino acids, of which 8 are cysteines (Joosten et al., 1997b). Although the AVR5 elicitor has not been purified, crude preparations of AVR5 have been used to study *Cf-5*—dependent responses. It has been suggested that G-proteins are involved in recognition of the AVR5 elicitor and the subsequent initiation of defense responses in MM-Cf5 tomato (Vera-Estrella et al., 1994; Xing et al., 1997). The *Cf-5* resistance gene is, however, not a G-protein–linked receptor however (Table1.2).

Outline of this thesis

At the start of the research described in this thesis, no resistance genes had been cloned. As resistance genes were proposed to encode the receptors for their matching elicitors, it was hypothesized the biochemical isolation of the AVR9 receptor would result in isolation of the product encoded by *Cf-9* resistance gene. Thus, to characterize and isolate the receptor for the AVR9 elicitor, AVR9 was labeled with iodine-125 and used in binding studies. As described in ***chapter 2***, ^{125}I-AVR9 showed specific, saturable, and reversible high-affinity binding to plasma membranes isolated from leaves of both MM-Cf0 and MM-Cf9 genotype. Binding

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a gene family and homologues of the *Cf-9* resistance gene are present in both resistant and susceptible tomato genotypes. Two new hypotheses were developed. The first hypothesis predicted that not only the *Cf-9* resistance gene, but also homologues of the *Cf-9* gene could encode the high-affinity binding site for AVR9. Only the protein encoded by *Cf-9* itself, designated CF-9, would subsequently initiate the signal transduction cascade resulting in HR. The second hypothesis predicted that the AVR9 binding site was not encoded by CF-9, nor by its homologues. The binding site would bind AVR9 and subsequently recruit the CF-9 protein to initiate HR. Experiments were designed to prove or reject these hypotheses.

A prerequisite for both hypotheses was to show that the high-affinity binding site for AVR9 plays a role in the initiation of HR (necrosis-inducing activity), showing that it is indeed the AVR9 receptor. Therefore, amino acids of AVR9 were identified that are required for its necrosis-inducing activity and the correlation between necrosis-inducing activity and affinity to the binding site was studied. The potato virus X expression system allowed direct analysis of amino acids of AVR9 required to initiate HR. In ***chapter 3***, amino acids of AVR9 important for its necrosis-inducing activity are identified. Based on the results presented in chapter 3, a number of mutant AVR9 peptides were selected to use in competition binding studies. ***Chapter 4*** describes the results of binding studies with mutant AVR9 peptides. Peptides were isolated from *N. clevelandii,* inoculated with PVX::*Avr9* derivatives, or chemically synthesized. Most of the peptides showed a positive correlation between their affinity to the membrane-localized binding site and their necrosis-inducing activity in MM-Cf9 tomato. This suggests that the AVR9 binding site is required to initiate the HR. In ***chapter 5*** it was tested whether CF-9 or CF-9—homologues are required for binding of AVR9. Therefore, binding of AVR9 to membranes of a series of solanaceous and non-solanaceous plant species was determined. These species were also tested for the presence of *Cf-9*-homologues. Also, Arabidopsis and tobacco, transformed with the *Cf-9* resistance gene, were tested for AVR9 binding. A correlation between the presence of *Cf-9* or *Cf-9* homologues and the high affinity binding site for AVR9 was not found. Based on the presented results, models for AVR9 perception and the role of the *Cf-9* resistance gene in initiation of plant defense are proposed in ***chapter 6.*** Common features of the proposed models and recognition systems described in literature are discussed.

CHAPTER 2

A HIGH-AFFINITY BINDING SITE FOR THE AVR9 PEPTIDE ELICITOR OF *CLADOSPORIUM FULVUM* IS PRESENT ON PLASMA MEMBRANES OF RESISTANT AND SUSCEPTIBLE TOMATO

Miriam Kooman-Gersmann, Guy Honée, Guusje Bonnema, and Pierre J.G.M. De Wit.

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Abstract

The race-specific *Cladosporium fulvum* peptide elicitor AVR9, which specifically induces a hypersensitive response (HR) in tomato genotypes carrying the *Cf-9* resistance gene, was labeled with iodine-125 at the N-terminal tyrosine residue and used in binding studies. ^{125}I-AVR9 showed specific, saturable, and reversible binding to plasma membranes isolated from leaves of the tomato cultivar Moneymaker without *Cf* resistance genes (MM-Cf0) or from a near-isogenic genotype with the *Cf-9* resistance gene (MM-Cf9). The dissociation constant was found to be 0.07 nM, and the receptor concentration was 0.8 pmol/mg microsomal protein. Binding was highly influenced by pH and ionic strength of the binding buffer and by temperature, indicating the involvement of both electrostatic and hydrophobic interactions. Binding kinetics and binding capacity were similar for membranes of the MM-Cf0 and MM-Cf9 genotype.

Introduction

Host specificity in plant—pathogen interactions has been described using the gene-for-gene model (Flor, 1971), in which the products of avirulence genes of a pathogen induce a hypersensitive response (HR) in plants that carry corresponding resistance genes. A typical feature of HR is the rapid death of a few cells that surround the infection site, which is thought to prevent further growth of the pathogen. The interaction between the fungal pathogen *Cladosporium fulvum* (syn. *Fulvia fulva*) and tomato is a model system to study the molecular basis of gene-for-gene—based resistance (De Wit, 1995).

The avirulence genes *Avr*4 (Joosten et al., 1994) and *Avr*9 of C. fulvum (Van den Ackerveken et al., 1992) have been cloned. Both avirulence genes occur as single-copy genes in the fungal genome. The *Avr*9 gene encodes a 63—amino acid peptide, which, after secretion, is processed by fungal and plant proteases to the mature 28—amino acid AVR9 elicitor peptide (Van den Ackerveken et al., 1993). The AVR9 elicitor is the only factor responsible for the induction of active defense responses in tomato plants that carry the complementary *Cf-9* resistance gene. This has been demonstrated by transferring the *Avr*9 gene to a race of *C. fulvum* virulent on *Cf-9* tomato genotypes, which resulted in avirulence (Van den Ackerveken et al., 1992). Disruption of the *Avr*9 gene in a race avirulent on *Cf-9* plants resulted in virulence (Marmeisse et al., 1993). Injection of the AVR9 peptide elicitor into leaves of a near-isogenic line of tomato cultivar Moneymaker, carrying the *Cf-9* resistance gene (MM-Cf9), resulted in a local necrotic response at the site of injection. The intensity of necrosis in the injected leaf area correlates with the concentration of the elicitor (Van den Ackerveken et al., 1992).

Recently, the *Cf-9* resistance gene of tomato, which is complementary to the *Avr*9 avirulence gene of *C. fulvum*, has been cloned and sequenced (Jones et al., 1994). The *Cf-9* gene belongs to a clustered gene family which maps at chromosome 1. Although members of the *Cf-9* gene family are expressed in leaves of both resistant and susceptible tomato genotypes, only genotypes that are resistant to strains of *C. fulvum* harboring the *Avr*9 gene, carry a functional *Cf-9* resistance gene (Jones et al., 1994). The amino acid sequence deduced from the *Cf-9* gene predicts an

extracytoplasmic, membrane-anchored glycoprotein with 28 extracellular leucine-rich repeats (LRRs).

Several studies have shown that the response of plants to non race-specific fungal elicitors is mediated through receptor proteins that are localized on the plasma membrane of the plant. Recently, receptors have been identified for an oligopeptide elicitor from *Phytophthora sojae* (Nürnberger et al., 1994) and for a glycopeptide elicitor from yeast (Basse et al., 1993). Binding of these elicitor peptides to membrane—localized binding sites is species-specific and correlates with the induction of defense-related plant responses. This correlation indicates that these receptors play an important role in the initiation of plant defense. Binding proteins have also been identified for several oligosaccharides derived from fungal cell walls (Cosio et al., 1988) and for the fungal toxins victorin from *Cochliobolus victoriae* (Wolpert et al., 1989) and fusicoccin from *Fusicoccum amygdali* (De Boer et al., 1989).

Victorin and fusicoccin have binding sites in all higher plants tested (Marrè, 1979; Loschke et al., 1994). Victorin binding proteins have been isolated from oat cultivars that are either susceptible or resistant to the toxin. Thus, although these binding proteins seem to be important for the activity of victorin, they do not determine the specificity of the plant response (Navarre and Wolpert, 1995).

Thus far, no resistance gene—associated receptors for race-specific peptide elicitors have been identified. Although the *Cf-9* resistance gene of tomato and the corresponding *Avr9* avirulence gene of *C. fulvum* have been cloned, the mechanism of perception of the race-specific AVR9 elicitor by *Cf-9*—resistant tomato plants is still unclear. To reveal whether a receptor for the AVR9 race-specific elicitor is present and to characterize this receptor, we performed binding studies with the AVR9 peptide. The peptide was radiolabeled with iodine-125 at its N-terminal tyrosine residue, and binding to plasma membranes of tomato leaves was studied. In this study we report the presence of a single class of high-affinity binding sites for the AVR9 elicitor on plasma membranes of tomato with and without the *Cf-9* resistance gene.

Results

Radiolabeling of the AVR9 peptide and optimization of binding conditions

The 28—amino acid AVR9 peptide was labeled by lactoperoxidase treatment, introducing radioactive iodine-125 into metapositions of the phenolic hydroxyl group of the Tyr-1 residue. Iodination did not affect the specific necrosis-inducing activity of the AVR9 peptide, as was shown by injection of non—radioactively iodinated AVR9 into leaves of MM-Cf9 (results not shown). Incubation of membrane preparations from MM-Cf9 tomato leaves with ^{125}I-AVR9 resulted in specific binding to microsomal membranes and enriched plasma membranes obtained by two-phase partitioning, as shown in Table 2.1. Plasma membranes showed ~10-fold higher binding capacity per milligram of protein compared with microsomal membranes, which indicates that the binding site is localized on the plasma membrane.

Plasma membranes that were obtained by reextraction of the lower phase of the two-phase system were less pure and showed less binding of ^{125}I-AVR9 than did the plasma membranes from the first extraction. The amount of bound radioligand increased linearly with the amount of plasma membrane in the range of 0 to 50 µg of membrane protein (results not shown). From Figure 2.1A, it is clear that binding is strongly temperature dependent, with slow binding at 4°C and fast binding at

Table 2.1 Isolation of tomato membranes by two-phase partitioning and binding of AVR9

Fractions	Yield (µg protein/g fresh weight)[a]	Specific H^+-ATPase Activity (nmol Pi/mg protein/min)[a]	Specific binding of ^{125}I-AVR9[a,b] (cpm)
Microsomal membranes	1621 ± 402	13 ± 3	1879[c]
Plasma membranes	30 ± 7	109 ± 13	16442 ± 2782
Membranes reextracted from lower phase	36 ± 12	63 ± 35	7466 ± 2898

[a] Data represent the average value of four independent membrane isolations.
[b] Binding of ^{125}I-AVR9 (final concentration 2 x 10^{-10} M) to 1 µg of membrane protein.
[c] Data from one isolation.

higher temperatures. Equilibrium conditions were reached after ~60 min, when plasma membranes were incubated with ^{125}I-AVR9 at 37°C (Figure 2.1A), as opposed to 480 min at 24°C. At 45°C, the initial binding was faster, but the maximum binding capacity was lower than at 37°C. At 60°C, the initial binding was similar to the binding at 37°C, but it strongly decreased ~10 min after the addition of AVR9, suggesting that the binding site is proteinaceous and becomes inactivated at high temperatures. The proteinaceous nature of the binding site could be confirmed by incubation of the membranes with different proteinases (e.g., trypsin, proteinase K, and protease XXIII). They all abolished the binding capacity of the membranes after treatment for 2 hr (results not shown). The different proteinases did not affect the AVR9 peptide, as was confirmed by low pH polyacrylamide gel electrophoresis (PAGE), where no change in mobility was observed. Also, proteinase-treated AVR9 showed no change in necrosis-inducing activity after injection into MM-Cf9 leaves (data not shown).

Figure 2.1B shows that binding is optimal between pH 5.5 and 6.0, resembling the pH of apoplastic fluid of *C. fulvum*—infected tomato leaves (results not shown). Increasing the pH of the binding buffer to 7.0 lowered the binding capacity by >50%, whereas at pH 8.0 no binding was observed. Decreasing the pH from 5.5 to 4.5 resulted in a 50% reduction of binding. Binding of the AVR9 peptide was also influenced by the ionic strength (*I*) of the assay buffer, as shown in Figure 2.1C. An increase in *I* from 0.014 to ~0.05 by the addition of NaCl, KCl, KI, $CaCl_2$, $MgCl_2$, or $MgSO_4$ resulted in a significant decrease in specific binding, whereas an increase of *I* to 0.164 by the addition of CaCl2 or MgCl2 practically abolished binding. Increasing the concentration of the phosphate buffer to 50 mM (*I* of 0.07) caused a decrease in binding capacity of the membranes by 25% (results not shown). The AVR9 peptide remained stable during incubation with membrane fractions for at least 2 hr, as was determined by nondenaturing, low pH PAGE of the reaction mixture, followed by autoradiography (results not shown). Based on the results described, standard equilibrium experiments were performed by incubating membranes with ^{125}I-AVR9 for 3 hr at 37°C in 10 mM phosphate buffer, pH 6.0, with 0.1% fatty acid—free BSA to reduce background binding.

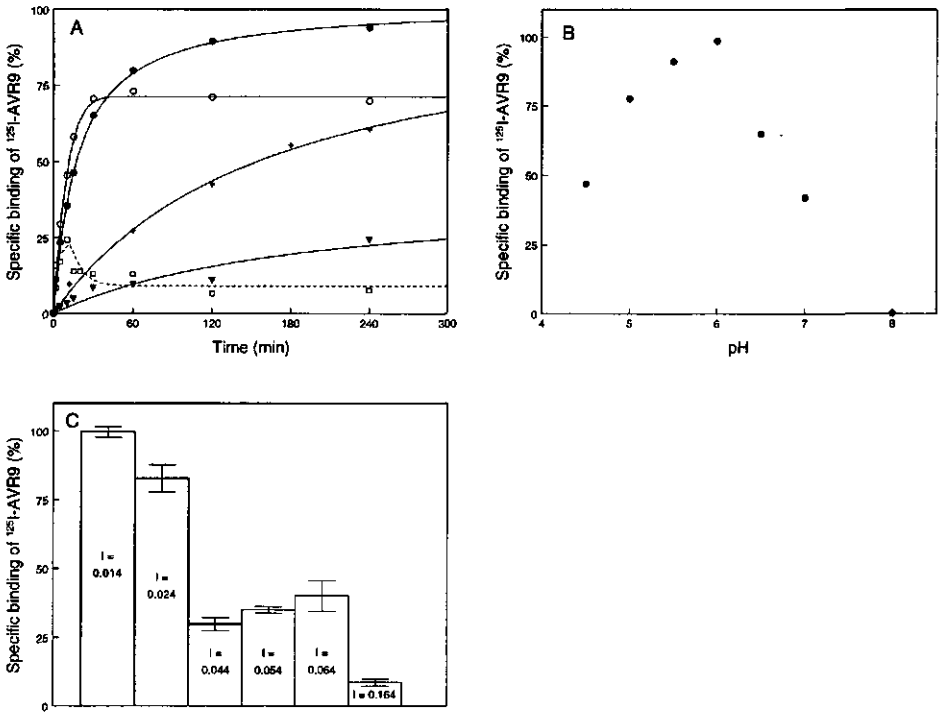

Figure 2.1 Effects of temperature, pH, and ionic strength on specific binding of ^{125}I-AVR9 to 1 µg of plasma membrane protein from leaves of MM-Cf9.

(A) Time courses of specific binding of ^{125}I-AVR9 (final concentration 2 x 10^{-10} M) to MM-Cf9 membranes at 4°C (▼), 24°C (+), 37°C (●) 45°C (O) and 60°C (□) (pH 6.0, and *I* of 0.014). Specific binding is total binding minus nonspecific binding, as determined by the addition of a 1000-fold excess of unlabeled AVR9. One hundred percent is 4.5 x 10^{-11} M ^{125}I-AVR9 bound.

(B) Effect of pH on the specific binding of ^{125}I-AVR9 (2 x 10^{-10} M) to MM-Cf9 membranes (at 37°C for 2 hr; *I* of 0.014). One hundred percent is 3.5 x 10^{-11} M ^{125}I-AVR9 bound.

(C) Effect of ionic strength (*I*) of the binding buffer on specific binding of ^{125}I-AVR9 (2 x 10^{-10} M) to MM-Cf9 membranes (at 37°C for 2 hr; pH 6.0) ($I = ½ \times \sum_i[m_i * z_i]$, where m_i and z_i are, respectively, the molality and the charge of the *i*th ion in the solution). Error bars give the standard error of the mean. An *I* of 0.014 represents binding in 10 mM phosphate buffer, no salts added; *I* of 0.024, 10 mM NaCl, KCl or KI added; *I* of 0.044, 10 mM CaCl2 or MgCl2 added; *I* of 0.054 10 mM MgSO4 added; *I* of 0.064, 50 mM KCl or KI added, *I* of 0.164, 50 mM CaCl2 or MgCl2 added. One hundred percent is 3.5 x 10^{-11} M ^{125}I-AVR9 bound.

All data represent the average of two measurements. Duplicate experiments gave comparable results.

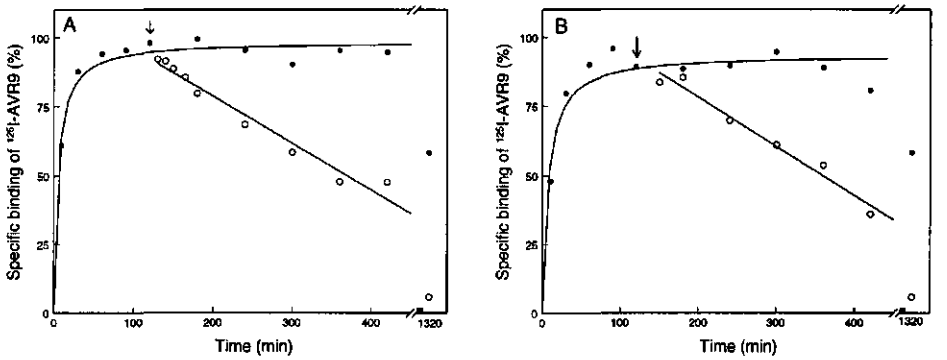

Figure 2.2 Time course of specific binding and displacement of ^{125}I-AVR9 to plasma membranes from MM-Cf9 and MM-Cf0.

(A) Binding to 2 µg of protein from plasma membrane fractions of MM-Cf9. Binding was initiated by adding ^{125}I-AVR9 to a final concentration of 7 x 10^{-10} M Displacement (O) was initiated by the addition of 7 x 10^{-7} M unlabeled AVR9 at a t of 120 min (arrow), whereas binding (●) indicates no addition of unlabeled AVR9.

(B) Binding to 2 µg of protein from plasma membrane fractions of MM-Cf0. Binding and displacement are as given in (A).

Characterization of the AVR9 binding site in tomato membranes

Kinetic analyses of ^{125}I-AVR9 binding to MM-Cf9 tomato membranes showed that at 37°C binding was half-maximal after 15 min, whereas an equilibrium between association and dissociation was reached after 60 min (Figure 2.2). Nonspecific binding, as determined by adding a 1000-fold excess of unlabeled AVR9 at the start of the binding experiment (t of 0 min), was ~10% of the total binding (results not shown). Dissociation of bound ^{125}I-AVR9 was accomplished by the addition of a 1000-fold excess of unlabeled AVR9 when binding was at equilibrium (t of 120 min). Complete dissociation of the binding complex was established after overnight incubation (Figure 2.2). Specific binding was reduced by ~50% after overnight incubation. This reduction may have been caused by instability of the binding site at 37°C. The slow dissociation rate constant (K_{off} of 5 x 10^{-5}/sec) indicates that the binding site has a high affinity for AVR9.

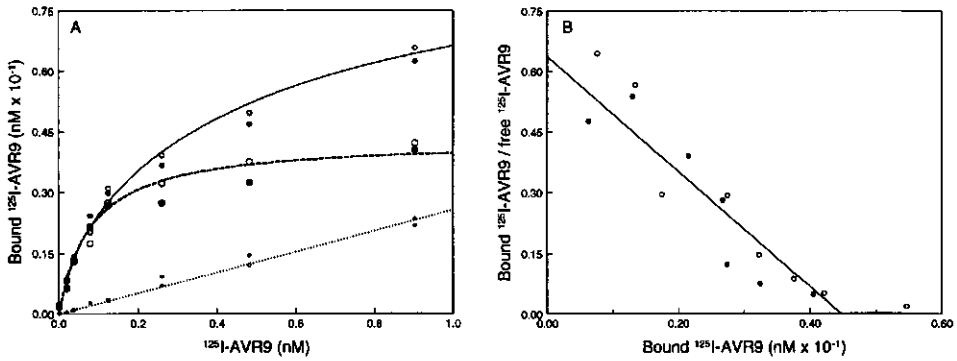

Figure 2.3 *Saturation of the AVR9-binding site in tomato membranes.*

(A) Microsomal membranes of MM-Cf0 (○) and MM-Cf9 (●) tomato plants were incubated with increasing amounts of ^{125}I-AVR9. The solid line represents total binding, and the dotted line represents nonspecific binding as determined by the addition of a 1000-fold excess of unlabeled AVR9. The dashed line represents specific binding (total minus nonspecific binding). The data represent one experiment, which is representative of six independent experiments.

(B) Scatchard plot of specific binding data derived from (A).

To characterize further the AVR9 binding site, microsomal membranes or plasma membranes were incubated with increasing amounts of ^{125}I-AVR9. As shown in Figure 2.3A, saturation of the binding sites was achieved at a concentration of ~0.5 nM AVR9. Linearization of the data in a Scatchard plot showed the presence of a single class of binding sites (Figure 2.3B). The dissociation constant (K_d) was calculated to be 0.07 nM by Scatchard analysis. The apparent concentration of the binding sites (R_t) was calculated to be 0.8 pmol/mg of membrane protein for microsomal membrane fractions and 7 pmol per milligram of protein for enriched plasma membrane fractions. Enriched plasma membrane fractions also have a 10-fold higher specific H^+-ATPase activity compared with microsomal membranes (Table 2.1). These results confirm that the binding site is localized on the plasma membrane.

Specificity of AVR9 binding to membranes of tomato genotypes with and without the Cf-9 *resistance gene*

No significant difference in the binding kinetics or binding capacity was observed between membranes of the resistant (MM-Cf9) and susceptible (MM-Cf0; without *Cf* resistance genes) tomato genotypes. Saturation experiments were performed with membranes of each of the two genotypes by using varying binding conditions. Significant differences in K_d or R_t between MM-Cf9 and MM-Cf0 plants were never observed (Figures 2.3A and 2.3B). Also, association and dissociation kinetics of binding of AVR9 to MM-Cf0 membranes, shown in Figure 2.2B, were identical to the kinetics of AVR9 binding to MM-Cf9 membranes (Figure 2.2A).

To correlate specific binding to the biological activity of the ligand (the AVR9 elicitor only induces HR in leaves of tomato genotypes that contain the *Cf-9* resistance gene), competition analyses were performed using non—radioactively iodinated AVR9, native and reduced AVR9, AVR4 and two small antifungal peptides. The latter four peptides do not induce necrosis upon injection into leaves of MM-Cf9. In competition assays, using either native AVR9 or non—radioactively iodinated AVR9 (data not shown) as competitors, an apparent K_d of ~0.2 nM was observed for both peptides. Thus, iodination barely affects binding properties of AVR9. In heterologous competition experiments, the antifungal cysteine-rich peptides of radish (RsAFP1; Terras et al., 1992;) and *Mirabilis jalapa* (MjAMP2; Cammue et al., 1992) and the AVR4 elicitor of *C. fulvum* (Joosten et al., 1994) did not compete for the AVR9 binding sites, as shown in Figure 2.4. Reduction of the disulfide bonds of the AVR9 peptide abolished the necrosis-inducing activity of the peptide and resulted in a ~1000-fold decrease in affinity (Figure 2.4). The observed K_d values vary somewhat between different competition experiments. This may have been due to loss of the very pure AVR9 competitor peptide at low concentrations, caused by nonspecific binding of AVR9 to incubation vials. Results of the binding experiments with different competitor peptides were similar for membranes isolated from leaves of MM-Cf9 and MM-Cf0 tomato plants (results not shown).

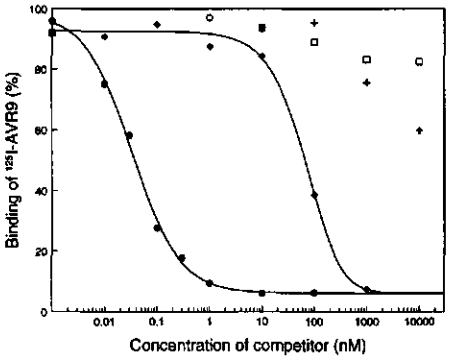

Figure 2.4 Competition of ^{125}I-AVR9 binding to plasma membrane fractions of MM-Cf9 tomato plants.

Binding was performed in 0.5 mL of binding buffer with a final concentration of 1 x 10^{-11} M ^{125}I-AVR9. Competitor peptides are as follows AVR9 (●), reduced AVR9 (◆), AVR4 (O), RsAFP1 (+) and MjAMP2 (□)

Discussion

A binding site for AVR9

Although the AVR9 elicitor only induces HR in tomato plants that carry the *Cf-9* resistance gene, high-affinity binding sites for AVR9 are present on plasma membranes of both the resistant (MM-Cf9) and susceptible (MM-Cf0) tomato genotype. No difference in K_d or R_t was observed between membranes of genotypes with or without the *Cf-9* resistance gene. The observed K_d value of 0.07 nM is low compared with K_d values of ligands binding to other plasma membrane-localized receptors in plants. Chitin fragments and a glycopeptide derived from yeast invertase bind to tomato membranes with K_d values of 23 and 3.3 nM, respectively (Basse et al., 1993; Baureithel et al., 1994), whereas a hepta-ß-glucoside from *P. sojae* was found to bind to soybean membranes with a K_d of 0.75 nM (Cheong and Hahn, 1991). A 13—amino acid peptide derived from the 42-kD glycoprotein elicitor from *P. sojae* binds to a receptor in parsley membranes with a K_d of 2.4 nM (Nürnberger et al., 1994).

In mammalian systems, high-affinity binding sites with K_d values in the picomolar range have been reported frequently. K_d values of 1 to 60 pM have been observed for binding of transforming growth factor-ß (TGF-ß) to cell surface receptors (Segarini et al., 1987). Very high affinities have also been found for the binding of inhibitors to enzymes. The ribonuclease inhibitor, a protein built entirely of LRRs, binds ribonuclease with a K_d of 1 to 70 fM (Hofsteenge, 1994). Binding of AVR9 is slow, with half-maximal binding after ~15 min at 37°C. A slow association rate is often found in combination with a low K_d (K_{off}/ K_{on}) value and is in agreement with the very low K_{off} of 5 x 10^{-5}/sec. The strong effect of temperature on binding of AVR9 to its binding site suggests the involvement of hydrophobic interactions, whereas the effects of pH and ionic strength suggest the involvement of electrostatic forces on binding as well. Histidine, of which two residues are present in the AVR9 peptide, has a pK value of 6. At pH values above 6 the histidine residues become deprotonated, which may cause the negative effect on AVR9 binding.

The concentration of AVR9 required to cause necrosis when injected into leaves of MM-Cf9 tomato plants is about 3 x 10^{-7} M, which is ~4000-fold higher than the K_d we have calculated from the binding studies. The apparent discrepancy between the *in vitro* and *in vivo* results may be due to the fact that the appearance of necrosis can be seen as a final step in the active defense response, which depends on physiological and environmental conditions. A similar phenomenon has been observed for fusicoccin, which invokes proton excretion in higher plants at a concentration of ~10^{-7} M, whereas its high-affinity receptors have reported K_d values of around 10^{-9} M (Basel et al., 1994). It is possible that the observed discrepancy occurs because binding of AVR9 to membranes is highly influenced by salt concentration and is optimal at low ionic strength. Not much is known about the ionic strength of the apoplast, and apparent K_d values may therefore be different from intrinsic values. Also, the cell wall could function as an adsorbent and diffusion barrier for AVR9, resulting in a low actual concentration of AVR9 near the plasma membrane receptor.

Although the affinity of AVR9 to its binding site is much higher than the affinities found for other plant receptors, the R_t of 0.8 pmol/mg microsomal protein is in the same range as that found for other membrane-bound receptors present in plants. In tomato, the reported R_t values vary from 19 fmol/mg microsomal protein for a

glycopeptide elicitor (Basse et al., 1993) to 2.45 pmol/mg microsomal protein for chitin fragments (Baureithel et al., 1994).

The observed binding site specifically binds native AVR9, barely binds reduced AVR9, and does not bind other small cysteine-rich peptides. Therefore, we suggest that the binding site is a functional receptor for AVR9 in resistant tomato genotypes. Because plasma membranes from MM-Cf0 plants also bind, this implies that there are AVR9 binding sites with similar binding characteristics but with potentially different functions.

Recently, the three-dimensional structure of the AVR9 peptide has been resolved by ^{1}H-NMR (Vervoort et al., 1997). The AVR9 peptide consists of a triple-stranded anti-parallel ß-sheet with three disulfide bridges. Based on its tertiary structure, the AVR9 peptide can be grouped into a superfamily of structurally related peptides with a common cystine-knot motif, which is found in several proteinase inhibiting peptides, various ion channel blockers (Pallaghy et al., 1994) and mammalian growth factors, such as TGF-ß (Isaacs, 1995). The tertiary structure of the AVR9 peptide is most closely related to the potato carboxypeptidase inhibitor (Chang et al., 1994). Whether this common motif also has functional implications remains to be determined.

Does CF-9 bind AVR9?

Tomato plants containing the *Cf-9* gene are resistant to AVR9-producing races of *C. fulvum*. Furthermore, the CF-9 protein contains 28 LRRs of 24 amino acids and is likely to be localized on the outer surface of the plasma membrane, with only 21 amino acids of the protein located intracellularly (Jones et al., 1994). LRRs were shown to be involved in protein-protein interactions, including binding of different types of protein ligands (Kobe and Deisenhofer, 1994). The suggestion that the CF-9 protein is directly involved in AVR9 perception, therefore seems plausible. Because the *Cf-9* gene belongs to a gene family, the CF-9 protein as well as its homologs could be involved in AVR9 binding. High-affinity AVR9 binding sites were found to be present on membranes of MM-Cf0 and MM-Cf9 tomato plants and these tomato genotypes contain members of the *Cf-9* gene family (Jones et al., 1994). Possibly the observed high-affinity binding sites are CF-9-like proteins. However, we cannot conclude that the functional CF-9 protein binds AVR9, and therefore additional molecules or proteins may still be involved in perception of AVR9.

Several systems have been described in which partly homologous proteins are required to give a biological response. The self-incompatibility systems of Arabidopsis and *Brassica* species have two partly homologous genes, the S locus glycoprotein (*SLG*) gene and the S locus receptor kinase (*SRK*) gene, which share sequence homology. Both *SLG* and *SRK* are required to give the incompatible phenotype (Nasrallah et al., 1994). A similar mechanism might be required for CF-9 and its homologs. Mechanisms for dimerization of partly homologous receptors also have been described for mammalian receptors, such as the TGF-ß receptors I and II, two distantly related receptors (Wrana et al., 1994). TGF-ß binds directly to receptor II and is then recognized by receptor I, which is recruited into the complex. Possibly, CF-9 homologs are required for AVR9 binding but do not give HR without the presence of the functional CF-9 protein (De Wit, 1995).

The involvement of the CF-9 protein in AVR9 perception will be studied in more detail by performing AVR9 binding experiments with membranes from different plant species and with membranes from tobacco and Arabidopsis plants, which have been transformed with the *Cf-9* resistance gene (chapter 5). Tobacco and Arabidopsis plants transformed with *Cf-9* will be used as representatives of a solanaceous plant and a non-solanaceous plant, respectively.

All known *C. fulvum* races that are virulent on tomato genotypes carrying the *Cf-9* resistance gene lack the *Avr*9 gene, and accordingly, they lack the AVR9 peptide. No virulent races of *C. fulvum*, producing inactive or less active AVR9 peptides, which might be used in binding studies, have yet been found in nature. Therefore, modified AVR9 peptides with increased or decreased necrosis-inducing activity have been designed (chapter 3). They have been used in heterologous competition experiments to characterize the AVR9 binding site in more detail (chapter 4).

Material and methods

Plant material

Leaves from the tomato (*Lycopersicon esculentum*) cultivar Moneymaker, without Cf resistance genes (designated MM-Cf0) and the near-isogenic genotype MM-Cf9 were harvested from 3-week-old plants that were grown in peat soil at 25°C under 16-hr-light conditions. Microsomal fractions and plasma membranes were isolated essentially

according to Sandstrom et al. (1987). All isolation steps were performed at 4°C. Briefly, large veins were removed from tomato leaves, and the leaves were ground in ice-cold buffer containing 25 mM Tris-HCl, pH 7.5, 250 mM sucrose, 3 mM EDTA, 10 µg/mL fatty acid–free BSA, and 1 mM phenylmethylsulfonyl fluoride. Following filtration through two layers of Miracloth (Calbiochem, La Jolla, CA) and differential centrifugation, plasma membranes were purified from the microsomal membranes by aqueous two-phase partitioning (Larsson et al., 1987). For partitioning, three successive extraction steps were performed in a 6.5% polyethylene glycol–6.5% dextran separating system containing 4 mM KCl, 250 mM sucrose, and 5 mM KPi, pH 7.5.

After isolation, both the microsomal membrane fractions and the plasma membrane-enriched fractions were dissolved in 10 mM Tris-HCl, pH 7.5 and 250 mM sucrose and stored at -80°C. Table 2.1 shows that a 10-fold enrichment of the plasma membranes could be reached in three subsequent extraction steps of the microsomal membranes in the 6.5% dextran-polyethylene glycol two-phase system. By reextraction of the three lower phases of the system, more plasma membranes could be obtained, but in general these membranes were less pure as determined by the specific activity of the K^+-, and Mg^{2+}- dependent H^+-ATPase. Based on these results the standard purification procedure of plasma membranes was performed by three consecutive extractions on the 6.5% dextran-polyethylene glycol phase system, without reextraction of the lower phase. By using this procedure, approximately 70-80% of plasma membranes was lost during partitioning (Table 2.1).

To determine the purity of the plasma membranes, the plasma membrane-localized, vanadate sensitive, K^+- and Mg^{2+}-dependent H^+-ATPase activity was determined according to Ames (1966) in the presence of 0.0075% Triton X-100 and 1 mM ammonium molybdate. Background activity was determined in the presence of 1 mM vanadate and subtracted from the total activity.

Purification and iodination of the AVR9 peptide

The 28–amino acid AVR9 peptide was isolated from apoplastic fluid, which was obtained from leaves of plants of the tomato genotype MM-Cf5 infected by race 5 of *C. fulvum*, as described by De Wit and Spikman (1982). Purification of the peptide was performed essentially according to the method described for the 34–amino acid AVR9 peptide from culture filtrate of a transformant of *C. fulvum* that constitutively produced AVR9, omitting the reversed-phase HPLC purification step (Van den Ackerveken et al., 1993). Briefly, high–molecular weight proteins were pelleted by overnight precipitation in 50% acetone, and then centrifugation. The supernatant was collected, and acetone was removed in a rotary evaporator at 40°C. The sample was further purified by cation exchange chromatography on a CM-Sephadex C-25 column (Pharmacia Biotech, Uppsala, Sweden), which was eluted using a pH gradient, followed by desalting of the active fractions on a Sep-Pak C18 cartridge (Waters, Milford, MA). Finally, cation exchange chromatography was performed on an MA7S column (7.8 x 50 mm, Bio-Rad), which was eluted with an NaCl gradient, and fractions were desalted once more on a Sep-Pak C18 cartridge before further use. The

peaks were analyzed by low-pH PAGE (Reisfeld et al., 1962), and the amount of purified 28—amino acid AVR9 peptide was determined by OD_{280} measurement by using a molar extinction coefficient of 1640, as determined by the Genetics Computer Group (Madison, WI) sequence analysis software package.

The N-terminal tyrosine residue of the purified elicitor was radiolabeled with iodine-125 by lactoperoxidase treatment (ANAWA laboratories Inc., Zürich, Switzerland), and the mono-iodinated peptide was isolated by reversed-phase HPLC, lyophilized, and stored at -20°C. The specific activity was 2200 Ci/mmol.

Competitor peptides

The 36—amino acid antifungal peptide MjAMP2 from *Mirabilis jalapa* (Cammue et al., 1992), and the 45—amino acid peptide RsAFP1 from radish (*Raphanus sativus*) (Terras et al., 1992) were a kind gift of W.F. Broekaert (F.A. Janssens Laboratory of Genetics, Catholic University of Leuven, Belgium). The 106—amino acid AVR4 elicitor peptide from *C. fulvum* (Joosten et al., 1994) was kindly provided by M.H.A.J. Joosten (Wageningen Agricultural University, Wageningen, The Netherlands). Reduction of the AVR9 peptide was performed by incubating 100 µg of AVR9 peptide in 0.1 M Tris-HCl pH 8.8, 1 mM EDTA, 6 M Guanidine-HCl, and 0.1 M DTT. The denatured peptide was reisolated by reversed-phase HPLC.

Binding of AVR9 to microsomal membranes and plasma membranes

Membranes were resuspended in 90 µL of binding buffer (10 mM phosphate buffer, pH 6.0, 0.1% BSA) and preincubated at 37°C for 20 min. Binding was initiated by the addition of 10 µL of different concentrations of ^{125}I-AVR9, followed by incubation under gentle shaking at 37°C in a water bath for 3 hr. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled AVR9. For competition experiments, membranes were suspended in 480 µL of binding buffer to which 10 µL 5×10^{-9} M ^{125}I-AVR9 and 10 µL of the competitor peptide were added. The membranes were not preincubated at 37°C but were kept on ice until both the iodinated AVR9 and the competitor peptide were added to ensure that no binding occurred until both peptides were present.

Glass fiber filters (Whatman GF/F) were soaked for 1 to 2 hr in 0.5% polyethylenimine, transferred to a filtration manifold (Millipore, Bedford, MA), and washed with 5 mL of H_2O, and 2 mL of binding buffer. Filtration of the samples was carried out at 10^4 Pascals and filters were subsequently washed with 12 mL of binding buffer. The filters were transferred to scintillation vials and 3 mL of LumaSafe Plus (LUMAC.LSC B.V., Groningen, The Netherlands) was added. The radioactivity was counted in a scintillation counter (model LS-6000 TA; Beckman Instruments).

Proteinase treatment of membranes

Enriched plasma membrane fractions were incubated with 0.1 mg of proteinase K from *Tritirachium album* (Merck, 124568), protease XXIII from *Aspergillus oryzae* (Sigma, P4032), or trypsin from bovine pancreas (Sigma, T8642). For this, 1 µg of membrane protein was incubated for 2 hr at 25°C at pH 7.5, with the various active or heat inactivated (control) proteinases, and immediately tested for binding of AVR9. Binding was performed as described above. Incubation of 1 µg of the AVR9 peptide with 0.1 mg of the proteinases for 3 hr did not affect the stability of the peptide. Proteinase treated AVR9 showed the same specific necrosis-inducing activity after injection into MM-Cf9 leaves as untreated AVR9 peptide. Also, migration patterns on a low pH polyacrylamide gel were similar for both untreated and proteinase treated AVR9.

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CHAPTER 3

ASSIGNMENT OF AMINO ACID RESIDUES OF THE AVR9 PEPTIDE OF *CLADOSPORIUM FULVUM* THAT DETERMINE ELICITOR ACTIVITY

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Abstract

The AVR9 peptide of *Cladosporium fulvum* is an elicitor of the hypersensitive response in tomato plants carrying the *Cf-9* resistance gene (MM-Cf9). To determine the structure-activity relationship of the AVR9 peptide, amino acids important for AVR9 elicitor activity were identified by independently substituting each amino acid of AVR9 by alanine. In addition, surface-exposed amino acid residues of AVR9 were substituted by other amino acids. Activity of the mutant *Avr*9 constructs was studied by expressing the constructs in MM-Cf9 tomato plants using of the potato virus X (PVX) expression system and assessing the severity of necrosis induced by each PVX::*Avr*9 construct. This allowed direct identification of amino acid residues of AVR9 that are essential for elicitor activity. We identified amino acid substitutions that resulted in AVR9 mutants with higher, similar or lower elicitor activity compared to the wild-type AVR9 peptide. Some mutants had completely lost elicitor activity. A selection of peptides, representing different categories, was isolated and injected into leaves of MM-Cf9 plants. The necrosis-inducing activity of the isolated peptides correlated well with the necrosis induced by the corresponding PVX::*Avr*9 derivatives. Based on the necrosis-inducing activity of the mutant AVR9 peptides and the global structure of AVR9, we assigned sites in AVR9 that are important for its necrosis-inducing activity. We postulate that the 'hydrophobic β-loop' region of the AVR9 peptide is crucial for necrosis-inducing activity in tomato plants that carry the *Cf-9* resistance gene.

Introduction

Host specificity in plant-pathogen interactions has been described by the gene-for-gene model (Flor, 1971). This model postulates that resistance is regulated by specific recognition of avirulence factors of the invading pathogen by the plant (De Wit, 1992; De Wit et al., 1992). Although the interactions between plants and bacteria, viruses, nematodes, and fungi comply with the gene-for-gene model, details on the molecular interaction between avirulence and resistance gene products remain unclear. Recently, the structure of a number of plant resistance genes has been revealed, and their general features show striking similarities (Bent, 1996; Staskawicz et al., 1995). The avirulence genes, however, appear more variable, and knowledge of their structure and intrinsic function remains limited (De Wit, 1995; Dangl, 1994). This has hampered progress on the elucidation of the molecular mechanisms involved in gene-for-gene–based interactions.

So far, a number of bacterial avirulence genes have been cloned (Dangl, 1994; Pirhonen et al., 1996), but the activity of their encoded products remains elusive. This is most likely due to the fact that they are not extracellular, like fungal elicitor products, but are delivered directly into the plant cell by the type-III secretion system (Jones, 1997; Leister et al., 1996; Van den Ackerveken et al., 1996; Gopalan et al., 1996). The products of two viral avirulence factors have been studied in more detail. The coat protein (CP) of tobacco mosaic virus (TMV) triggers resistance in tobacco plants expressing the *N'* resistance gene. The elicitor-active parts of the CP have been studied extensively, and a domain within the TMV CP has been assigned whose structural integrity is crucial for recognition by the host (Taraporewala and Culver, 1996). The CP of potato virus X (PVX) triggers resistance in potato plants expressing the *Rx*1-gene (Bendahmane et al., 1995). As yet, few fungal avirulence genes encoding race-specific elicitors have been cloned. The NIP1 elicitor peptide of the barley pathogen *Rhynchosporium secalis* is a small race-specific elicitor that triggers defense responses, including the activation of the PR*Hv*-1 gene, in barley cultivars carrying resistance gene *Rrs* 1 (Rohe et al., 1995).

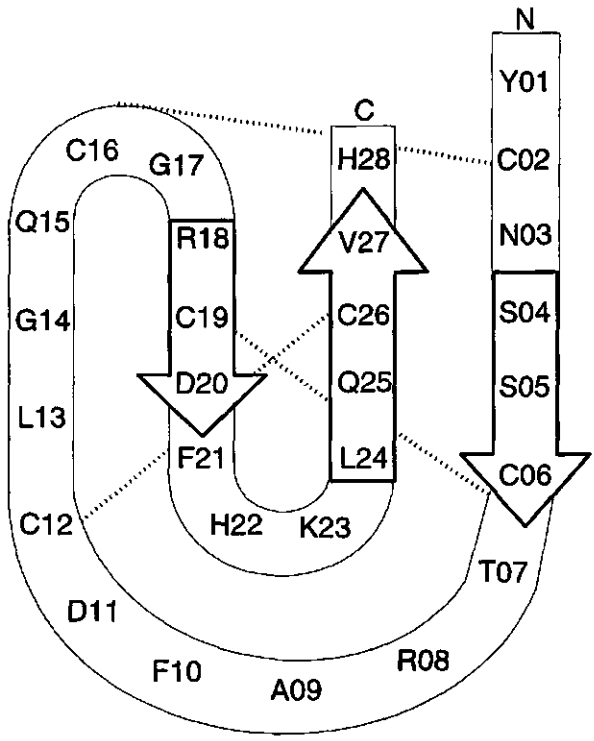


Figure 3.1 Schematic representation of the structure of AVR9, based on the common structure of cystine-knotted peptides (Figure adapted from Pallaghy et al., 1994).

The interaction between the leaf mold pathogen *Cladosporium fulvum* and its only host, tomato (*Lycopersicon esculentum*), is a well-established model system for studying gene-for-gene–based resistance (De Wit, 1995). An incompatible *C. fulvum*-tomato interaction is associated with the hypersensitive response (HR), which is thought to play a central role in disease resistance. HR is characterized by the rapid death of host cells adjacent to the site of fungal penetration and the activation of a variety of defense responses in the surrounding area (Dangl et al., 1996). The *C. fulvum* avirulence genes *Avr*4 and *Avr*9 have been cloned and their products, AVR4 and AVR9, have been characterized (Joosten et al., 1994; Van Kan et al., 1991; Van den Ackerveken et al., 1992). The AVR4 and AVR9 elicitors

trigger HR in tomato plants carrying the resistance genes *Cf-4* and *Cf-9*, respectively. The mature AVR4 elicitor consists of 86 amino acids, of which 8 are cysteine residues (Joosten et al., 1997). The AVR9 elicitor is encoded as a 63—amino acid pre-propeptide. Like the AVR4 peptide, AVR9 is secreted by the fungus when it infects of tomato plants. The mature AVR9 elicitor peptide consists of 28 amino acids (Van den Ackerveken et al., 1993). Injection of the AVR9 peptide into leaves of cv. Moneymaker (MM-Cf0) tomato plants and leaves of a near-isogenic line carrying the *Cf-9* resistance gene (MM-Cf9), results in necrosis only in the MM-Cf9 genotype. The tomato *Cf*-resistance genes, *Cf-2* (Dixon et al., 1996), *Cf-4* (Jones et al., 1996), *Cf-4A* (Takken et al., 1997), *Cf-5* (Jones et al., 1996), and *Cf-9* (Jones et al., 1994), recently have been cloned and characterized. All CF-proteins are predicted to be extracytoplasmic with a transmembrane domain and a small cytoplasmic domain. *Cf-9* encodes an 863—amino acid protein containing 27 imperfect leucine-rich repeats (LRRs) and 22 potential glycosylation sites (Jones and Jones, 1996). Although the *Cf-9* resistance gene only confers resistance to races of *C. fulvum* expressing the corresponding *Avr*9 gene, a binding site for the AVR9 elicitor was found in both MM-Cf0 and MM-Cf9 genotypes (chapter 2). The definite role of the described binding site in AVR9-induced resistance in tomato plants carrying the *Cf-9* resistance gene is not yet understood.

^{1}H-NMR studies have resolved the global fold of the AVR9 peptide (Vervoort et al., 1997). The AVR9 peptide consists of three antiparallel β-sheets and one extended loop as is schematically represented in Figure 3.1. The AVR9 peptide has a very compact and stable structure due to three disulfide bridges that are arranged in a knot. The elicitor has structural homology to peptides belonging to the group of cystine-knotted peptides (Pallaghy et al., 1994; Isaacs, 1995). A generally applied strategy to decode the structure-activity relationships of peptide or protein domains, is alanine-scanning mutagenesis (Cunningham and Wells, 1989; Sahm et al., 1994; Reidhaar-Olson et al., 1996). This involves a systematic study in which each single amino acid residue of a peptide is independently substituted by alanine. More detailed information on the amino acid residues that are important for biological activity of a peptide can be obtained by selectively substituting solvent-exposed amino acids by other amino acid residues. We have performed a combination of alanine-scanning mutagenesis and selective amino acid substitutions to study the structure-activity relationships of the AVR9 peptide.

The PVX expression system has been used to transiently express heterologous genes in solanaceous plants (Chapman et al., 1992). Expression of the wild-type *Avr*4 or the wild-type *Avr*9 gene of *C. fulvum* by use of the PVX expression vector causes systemic necrosis only in tomato plants carrying the *Cf-4* or the *Cf-9* resistance gene, respectively (Joosten et al., 1997; Hammond-Kosack et al., 1995), but not in MM-Cf0 or tobacco plants. In this study, mutants of the *Avr*9 gene were produced by site-directed mutagenesis (Horton and Pease, 1991) and expressed in MM-Cf9 plants using the PVX expression system. The elicitor activity of mutant AVR9 peptides was analyzed by detailed assessment of the severity of systemic necrosis induced in the mutant PVX::*Avr*9-infected plants, compared with the necrosis induced by wild-type PVX::*Avr*9. This approach allowed the assignment of amino acid residues within the AVR9 peptide that are important for its elicitor activity.

Results

*Necrosis induced by PVX::*Avr*9 constructs on MM-Cf9 plants*

MM-Cf9 plants infected with mutant PVX::*Avr*9 derivatives were screened for development of systemic necrotic lesions. The development of necrosis was followed over time for a period of 21 days, and symptoms were divided into six classes: 1 (–), 2 (+), 3 (++), 4 (+++), 5 (++++), and 6 (+++++). Figure 3.2A shows typical examples of plants with various degrees of necrosis. In class 1 (–), only mild mosaic symptoms caused by the PVX::*Avr*9 derivatives were visible on the infected MM-Cf9 tomato plants. In class 2 (+), the inoculated cotyledons of the plant died, and necrotic spots were observed at the tips of the inoculated compound leaves. Plants continued to grow, and occasionally small necrotic spots appeared on the older leaves. In class 3 (++), the cotyledons and inoculated leaves showed necrosis, and systemic spread of small necrotic spots was observed on all leaves of the plant. In class 4 (+++), spreading of both small and larger necrotic spots was observed throughout the plant, and occasionally, leaves turned completely necrotic. The apical meristem of these plants was not affected and therefore the plants continued to grow, while the older leaves eventually died. In class 5 (++++), plants showed complete systemic necrosis and died approximately 2 weeks after inoculation. The first necrotic symptoms appeared at 4 to 5 days after inoculation.

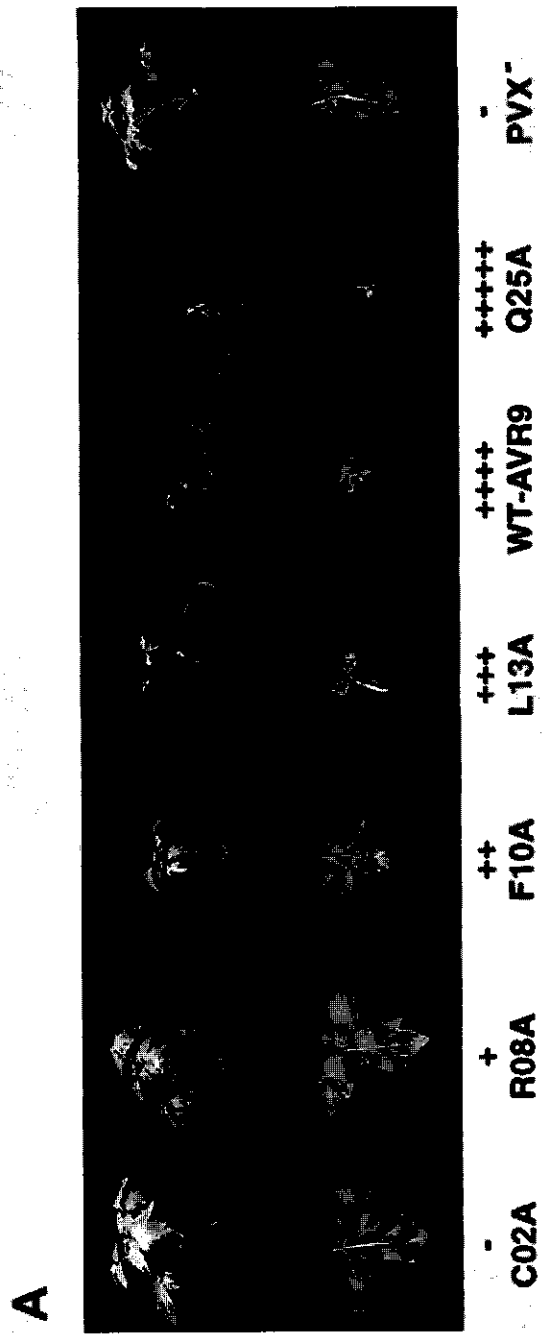
In class 6 (+++++), plants died within 10 days after inoculation, whereas the first necrotic symptoms appeared approximately 3 days after inoculation.

Symptoms were observed on plants inoculated 3 to 4 weeks after sowing. Inoculations with all mutant PVX::*Avr*9 constructs were repeated three to six times and were reproducible, i.e., the necrosis observed was always typical for each particular mutant compared to wild-type PVX::*Avr*9-infected plants. Growth conditions affected necrosis to some extent, but the differences in the necrosis-inducing activity between the mutants were consistent. Also, variation was observed when plants of a different age were used. Young plants died more rapidly than older plants, but again, the differences between the various mutant PVX::*Avr*9 constructs remained consistent.

Effect of amino acid substitutions on necrosis-inducing activity

The cystine-knotted structure of the AVR9 peptide, as schematically represented in Figure 3.1, shows three antiparallel ß-sheets, a short loop (F21-L24), and an extended loop (T07-R18). Disruption of the cystine-knotted structure by modification of any of the three disulfide bonds (C02-C16, C06-C19 or C12-C26), led to complete loss of necrosis-inducing activity of the peptide. Residues C16, C19 and C26 were not modified, because they were expected to give results identical to their counterparts C2, C6 and C12. Table 3.1 shows the symptoms observed after inoculation of MM-Cf9 plants with mutant PVX::*Avr*9 derivatives coding for AVR9 peptides with single alanine substitutions. Most alanine substitutions did not result in substantial differences in necrosis-inducing activity (+++ or ++++) compared to the wild-type PVX::*Avr*9 (++++). Substitution of the amino acids in the N-terminal part of the extended loop region, from position T07 to D11, greatly affected the necrosis-inducing activity of the AVR9 elicitor. The mutant PVX::*Avr*9 constructs, encoding amino acid residues T07, R08, A09, F10, or D11 substituted by alanine or valine (A09V), all had decreased necrosis-inducing activity (+ or ++) (Table 3.1). The short loop of AVR9, which comprises amino acids F21 to L24, is localized in the same region of the peptide as the amino acid residues T07 to D11 (Vervoort et al., 1997; Figure 3.1). Within this short loop, two mutants (F21A and L24A) had completely lost necrosis-inducing activity. Furthermore, two mutants outside the loop regions (N03A and H28A) had decreased necrosis-inducing activity. Substitution of glutamine (Q25A), which is located in the third β-sheet, resulted in an increase in necrosis-inducing activity (+++++) compared to wild-type AVR9 (++++).

In addition, a subset of amino acids of the AVR9 peptide, which are solvent-exposed, was substituted by different amino acid residues. The effect of these substitutions and of some double substitutions on the necrosis-inducing activity of their respective PVX::*Avr*9 derivative is shown in Table 3.2. Most of the additional PVX::*Avr*9 mutants showed similar effects on necrosis-inducing activity, as was found for the alanine mutants (Table 3.1). The significance of the extended loop region for AVR9 necrosis-inducing activity was confirmed by the R08S and F10S PVX::*Avr*9 constructs, because both resulted in a decrease of necrosis-inducing activity compared to wild-type AVR9. The R08K mutation resulted in a PVX construct with higher necrosis-inducing activity than the wild-type AVR9. This also was observed for the R18K mutation. Not only the F21A and L24A mutants, but also PVX::*Avr*9 derivatives encoding the F21S and L24S mutant peptides were inactive, confirming the importance of the F21 and L24 residues for AVR9 necrosis-inducing activity. The basic amino acids H22 and K23, which are located between these two crucial residues, were not important for necrosis-inducing activity of the AVR9 peptide. Both alanine substitutions (H22A and K23A) and substitutions by neutral amino acid residues (H22L and K23Q) retained activity (Tables 3.1 and 3.2). The necrosis-inducing activity of the double mutants, which showed up as cloned PCR products during sequencing, confirmed the results obtained with the single amino acid substitutions.


A
-
C02A
+
R08A
++
F10A
+++
L13A
++++
WT-AVR9
+++++
Q25A
-
PVX-

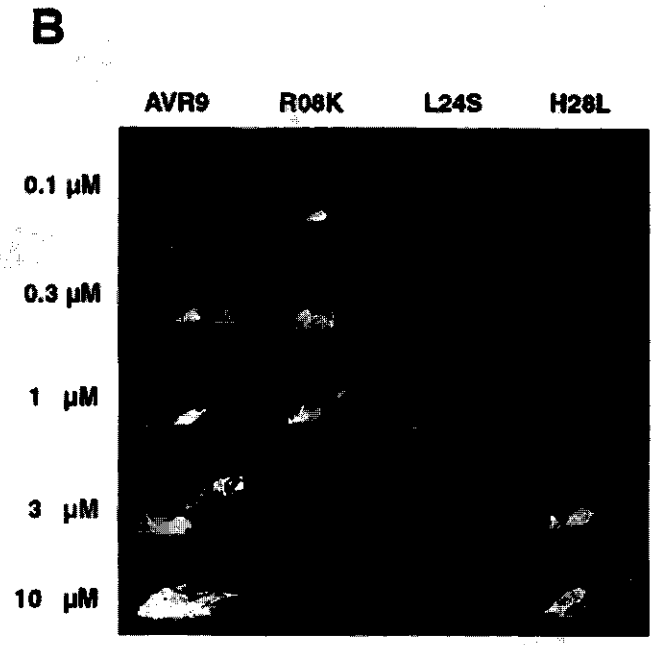


Figure 3.2 Necrosis-inducing activity of wild-type and mutant AVR9 peptides.

(A). Classes of necrosis induced by wild-type (class 5: ++++) and mutant PVX::*Avr*9 derivatives. A representative of each class has been selected, and their respective PVX::*Avr*9 constructs were inoculated on MM-Cf9 plants. Class 1(-) is represented by both a cysteine-mutant (C02A) and the PVX^{-}-vector, without the *Avr*9 gene. Photographs were taken at day 10 post inoculation.

(B). Necrosis-inducing activity of wild-type and mutant AVR9 peptides isolated from PVX::*Avr*9-inoculated *N. clevelandii*. Ten microliters of different concentrations of AVR9 peptides were injected near the midvein of the leaf. Necrosis was visible one day after injection and photographs were taken when the necrotic spot had dried 7 days after injection. Injections were repeated twice with similar results.

Table 3.1 Effect of alanine substitutions on the necrosis-inducing activity of AVR9, tested by inoculation of MM-Cf9 plants with the PVX::*Avr9* derivatives.

Mutant	Necrosis-inducing activity[a]	Mutant	Necrosis-inducing activity[a]
Wild Type	++++	L13A	+++
Y01A	+++	G14A	++++
C02A	-	Q15A	++++
N03A	++	G17A	+++
S04A	+++	R18A	++++
S05A	+++	D20A	+++
C06A	-	F21A	-
T07A	++	H22A	+++
R08A	+	K23A	++++
A09V	+	L24A	-
F10A	++	Q25A	+++++
D11A	++	V27A	++++
C12A	-	H28A	++

[a] The different classes of necrosis-inducing activity are 1 (-), 2 (+), 3 (++), 4 (+++), 5 (++++), and 6 (+++++) (shown in Figure 3.2A).

Table 3.2 Effect of the non-alanine substitutions on AVR9 necrosis-inducing activity, tested by inoculation of MM-Cf9 plants with the PVX::*Avr9* derivatives.

Mutant	Necrosis-inducing activity[a]	Mutant	Necrosis-inducing activity[a]
Wild Type	++++	D20N	++++
S04A + G17E	+++	F21S	-
R08S	+	H22L	+++
R08K	+++++	K23Q	+++
F10S	+	L24S	-
D11N	+++	Q25E	++++
Q15A + R18S	++++	Q25E + V27I	++++
L13A + C16stop	-	C26G	-
R18K	+++++	H28L	++

[a] The different classes of necrosis-inducing activity are 1 (-), 2 (+), 3 (++), 4 (+++), 5 (++++), and 6 (+++++) (shown in Figure 3.2A).

*Virus titer and elicitor activity of AVR9 peptides isolated from PVX::*Avr9*-infected* N. clevelandii

Several control experiments were performed to prove that the observed necrosis was not caused by a difference in PVX::*Avr9* virus titer but by a difference in the necrosis-inducing activity of the encoded peptides. A dilution series was made of sap of PVX::*Avr9*-infected *N. clevelandii* plants, including PVX derivatives containing the wild-type *Avr9* gene (++++) and the R08K (+++++) and H22L (+++) *Avr9* mutants. After inoculation of MM-Cf9 plants with 10- to 1000-fold—diluted sap, the severity of necrosis was assessed over time. Necrosis induced by the various dilutions, containing different virus titers, was similar, and differences between the mutants remained (results not shown). This indicates that the virus titer initially used in the undiluted sap was sufficient to ensure optimal infection of MM-Cf9 plants and that the necrosis-inducing activity was independent of the PVX virus titer. For several of the mutants, two independent PVX::*Avr9* constructs were made and inoculated on MM-Cf9 plants. Identical, independently made constructs always gave comparable results. A selection of mutant AVR9 peptides was isolated from PVX::*Avr9*—infected *N. clevelandii* plants. These included R08K, L24S, and H28L, representing peptides with, respectively, higher, no, or lower elicitor activity compared to wild-type AVR9. The peptides were purified, and the last purification step on reversed-phase HPLC resulted in single peaks for all the peptides (data not shown). Approximately 1 to 2 μg of wild-type or mutant AVR9 peptide could be isolated per inoculated *N. clevelandii* plant, even of the inactive mutant peptide L24S. Necrosis-inducing activity of the mutant AVR9 peptides was assayed by injection of a dilution series into leaflets of MM-Cf9 tomato plants. Figure 3.2B shows an injection series of the mutant and wild-type AVR9 peptides. Wild-type AVR9, isolated from PVX::*Avr9*-infected tobacco leaves, showed necrosis at a concentration of ~0.3 μM. The more active peptide in the PVX-based assay, R08K (+++++), induced necrosis at concentrations as low as ~0.1 μM, whereas the less active mutant in the PVX-based assay, H28L (++), required a concentration of ~3 μM or higher to induce necrosis. The inactive L24S peptide (–) did not induce necrosis at concentrations up to 10 μM. Hence, the results of the injection series with purified wild-type and mutant AVR9 peptides are comparable to the results obtained with the respective PVX::*Avr9* inoculations on MM-Cf9 plants.

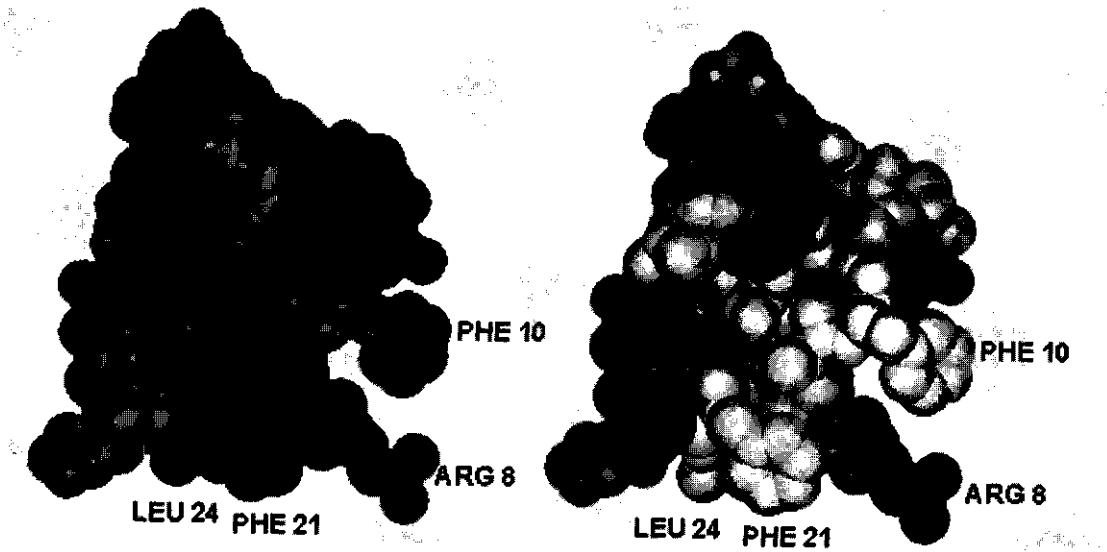


Figure 3.3 Surface views of the global structure of the AVR9 peptide (Vervoort et al., 1997). See back cover of this thesis for color representation. The left panel (bottom panel on the back cover) shows the effect of amino acid substitutions on the necrosis-inducing activity of AVR9. Residues representing necrosis-inducing activity of class 4, 5 and 6 (+++, ++++, +++++) are in light-blue, cystines are in yellow, residues representing necrosis-inducing activity of class 2 and 3 (+ and ++) are in pink and the F21 and L24 residues representing mutants that have lost elicitor activity (-), are in red. Residues T7-D11 are residues T12-D20 in Vervoort et al. (1997), because they used a 33–amino acid AVR9 peptide in their studies. The right panel (top panel on the back cover) shows the charge distribution of the AVR9 molecule. Positively charged residues are in blue, negatively charged residues are in red, polar residues are in green, hydrophobic residues are in yellow, and the glycine residue is in white.

Discussion

The PVX expression system: Ideal for structure-function analyses of AVR9.

Progress in determining active sites of cystine-knotted peptides has been hampered by the lack of a good expression system for these peptides, combined with the lack of a convenient bioassay (Olivera et al., 1994; Nielsen et al., 1996). We show that the PVX expression system is well suited for mutational analysis of the AVR9 peptide and for testing necrosis-inducing activity of AVR9 mutants on MM-Cf9 tomato plants.

We determined structure-activity relationships of the AVR9 peptide by alanine-scanning mutagenesis and expressed the mutant peptides directly in MM-Cf9 tomato plants by the PVX expression system. During systemic spread of PVX, we did not observe a shift in the necrosis-inducing activity of any of the mutant PVX::*Avr9* constructs. Therefore, we conclude that the PVX constructs remain stable during viral spread through the plant and that no loss of insert occurs, as has been described by Hammond-Kosack et al. (1995). Furthermore, between 1 and 2 µg of wild-type or mutant AVR9 peptide could be isolated per inoculated *N. clevelandii* plant, even for the inactive mutant peptide L24S. This confirms that the PVX expression system provides a reliable and reproducible method to determine elicitor-active regions of AVR9.

Effect of amino acid substitutions on the necrosis-inducing activity of AVR9

The PVX::*Avr9* derivatives carrying alanine substitutions in residues T7 to D11 showed a decrease in necrosis-inducing activity. This indicates that these first 5 amino acid residues of the large extended loop of AVR9 are important for the necrosis induction. These results are confirmed by the decrease or increase (R08K) in necrosis-inducing activity of the additional mutants in this region. Furthermore, the hydrophobic residues F21 and L24 in the small loop of AVR9 are crucial determinants of elicitor activity. This was shown by the complete loss of necrotic symptoms when these residues were substituted by alanine or serine. The two important sites in AVR9, T7 to D11 and F21 and L24, are in close proximity. This is shown in more detail in Figure 3.3, which represents the global structure of AVR9.

AVR9 has a hydrophobic region, consisting of the three residues F10, F21, and L24, which are in solvent-exposed positions. On one side, the hydrophobic amino acids are flanked by the basic residues H22 and K23, which do not seem essential for necrosis induction. The acidic residue D11 is located on the opposite side of the hydrophobic core. The hydrophilicity of the side-chain of D11 may be important for the structure or necrosis-inducing activity of AVR9, because substitution by asparagine (D11N) produced fairly strong necrosis (+++), whereas substitution by an alanine residue showed further reduction of necrosis (++). The basic R08 also flanks F10 and F21 (Figure 3.3). The R08K mutant showed an increase in necrosis-inducing activity compared to the wild-type AVR9, indicating that the hydrophobic part of this residue and its positive charge are involved in necrosis induction. Substitution of R08 to A or S led to a decrease in necrosis-inducing activity, confirming the importance of the characteristics of this residue. Even small changes in the extended loop of AVR9 influenced activity, as shown by the decrease in necrosis-inducing activity of the A09V mutant. Three mutant PVX::*Avr*9 derivatives, coding for amino acid substitutions outside the hydrophobic region of AVR9, (N03A, H28A and H28L), also showed reduced necrosis. Possibly, these substitutions led to perturbation of the global fold of AVR9. As expected, preventing formation of the cystine-knotted structure by disruption of one of the three disulfide bonds (C02-C16, C06-C19, or C12-C26) led to a complete loss of necrosis-inducing activity of the AVR9 peptide.

Comparison of AVR9 with structurally related peptides

AVR9 belongs to a family of structurally related peptides, that share a cystine-knot motif. The cystine knot motif has been observed in many apparently unrelated peptides, including growth factors (McDonald and Hendrickson, 1993), ω-conotoxins (Sevilla et al., 1993), the squash-seed family of protease inhibitors (Bode et al., 1989, Heitz et al., 1989), the sweet-taste suppresser gurmarin (Arai et al., 1995), and kalata B1, a cyclic plant peptide (Saether et al., 1995). Although some family members display similar biological activities and have comparable three-dimensional structures, they share very little sequence similarity. Where known, the peptides have a high affinity for their target proteins. Hence, it appears that this motif is able to accommodate a wide range of sequences and biological activities. For some of the cystine-knotted peptides, the active sites have been identified by chemical synthesis of analogues, followed by activity and binding analyses (Kim et al., 1994; Sato et al., 1993). Where known, the active sites of the

peptides, as of AVR9, are located in the solvent-exposed regions, never in the core cystine region (Kim et al., 1994; Le-Nguyen et al., 1993).

AVR9 structurally is most closely related to carboxypeptidase inhibitor (Vervoort et al., 1997). The active site of carboxypeptidase inhibitor is located in the C-terminal tail of the peptide and comprises Y-V-G, a sequence that is not present in AVR9. For the cystine-knotted trypsin inhibitors, the trypsin-binding loop comprises the amino acid residues P-R-I-L-M (Cliche et al., 1993), a site that also is absent in the AVR9 peptide. The P, I, and M residues of the trypsin inhibitors form a hydrophobic site. Peptidase inhibitors of *N. alata* are structurally somewhat less related to AVR9 (Nielsen et al., 1994). The typical trypsin-inhibiting region of these peptides comprises amino acid residues R and N, whereas the chymotrypsin-inhibiting region comprises amino acid residues L and N, which are present on an exposed loop of the protein. Again, these sites are not present in AVR9. So, although the structure of AVR9 closely resembles that of many peptidase inhibitors, AVR9 does not contain amino acid residues identical to the known inhibitory sites. In preliminary experiments with wild-type AVR9, no protease-inhibiting activity was found (results not presented). Additional assays are required to ascertain the lack of protease-inhibiting activity of AVR9.

In addition to protease-inhibitors, AVR9 is structurally related to several ion channel blockers or toxins. As yet, no toxic activity of AVR9 has been reported. The ω-conotoxin GVIA (ω-CTX GVIA) from *Conus geographus* venom binds to Ca^{2+} channels with very high affinity. For ω-CTX GVIA the amino acid residues important for binding and channel-blocking activity have been assigned by alanine-scanning (Kim et al., 1994). Most alanine substitutions do not affect the inhibiting activity of the peptide, except for substitution of K2, which leads to a 40-fold decrease of Ca^{2+} channel-inhibiting potential (Sato et al., 1993), and substitution of Y13, which leads to a dramatic 1000-fold decrease in Ca^{2+} channel-binding (Kim et al., 1994). The cystine-knotted structure of ω-CTX GVIA is required for its activity, because linearization renders the molecule inactive (Kim et al., 1994). This also holds for AVR9, in which linearization abolishes necrosis-inducing activity of the peptide (chapter 2).

For the cyclic cystine-knotted peptide kalata B1, no structure-activity data are available. One of the features of this peptide is the relatively large hydrophobic surface present on one side of the peptide (W02-P03-V04 and V18-G19,

representing 2 flanking loops). The elicitor-active site of AVR9 also has an exposed hydrophobic region comprising two flanking loops that contain the two phenylalanines and a leucine (F10, F21 and L24). For kalata B1 it is speculated that the flexible loop (residues 8 to 12, N-G-L-P-V) may be important for activity (Saether et al., 1995). Several other channel blocking peptides have exposed, hydrophobic amino acid residues flanked by positively charged residues, which are proposed to be part of the channel binding surface (Manoleras and Norton, 1994). These features are also found in AVR9, in which the F10, F21, and L24 residues are solvent exposed and flanked by the positively charged R08, H22 and K23 residues.

We conclude that, although the cystine-knot peptides share structural similarities their functional similarities are less obvious. The cystine-knot structure seems an energetically favorable structure that is well suited to exhibit inhibitory or channel-blocking activities. Next to their common structural features, the only additional similarity between AVR9 and the known cystine-knotted peptides is the presence of hydrophobic surface regions. The hydrophobic surfaces of the channel-inhibiting proteins are thought to embody the inhibiting regions. Although no inhibitory function of AVR9 is known yet, we speculate that the elicitor-active hydrophobic surface of AVR9 may not be involved only in necrosis induction but may reflect its intrinsic biological function as a pathogenicity factor. Analogous to other cystine-knotted peptides, AVR9 may exhibit an inhibitory or stimulating function in plant metabolism by interfering with a specific ion channel or nutrient pump. The *Avr9* gene is induced under nitrogen-limiting conditions (Van den Ackerveken et al., 1994), suggesting a role for AVR9 in amino acid metabolism or uptake. Although no pathogenicity function of AVR9 has been demonstrated yet, various reported extracellular elicitor peptides produced by *C. fulvum in planta* are likely to play a role in pathogenicity. This has been demonstrated for the ECP1 and ECP2 peptides of *C. fulvum* (Laugé et al., 1997). Possibly, these different extracellular peptides provide the fungus with a large array of possibilities to retrieve nutrients from the plant. The specific recognition of the AVR9 peptide by tomato plants carrying the *Cf-9* resistance gene shows the versatility of plants to recognize foreign peptides through a process of coevolution.

Material and methods

Cloning and mutagenesis

Standard molecular biological techniques were performed as described by Sambrook et al. (1989). Primer-directed mutagenesis of the *Avr*9 gene was performed by PCR overlap extension, essentially according to the protocol of Horton and Pease (1991). The wild-type PVX::*Avr*9 plasmid vector (Hammond-Kosack et al., 1995) was used as template. Primers were designed to introduce the chosen mutations into the target codons, as listed in Table 3.3 for alanine substitutions and Table 3.4 for other substitutions. All primers were 20-23 bp long, with a minimal calculated melting temperature (*Tm*) of 50 °C (*Tm* = 2(A-T) + 4(G-C), excluding the mutational mismatches). In the first mutagenesis step two PCR amplification reactions were performed, with the PVX::*Avr*9 plasmid vector as template DNA. In the first reaction we used the OX10-primer (5′-CAA TCA CAG TGT TGG CTT GC-3′, upstream of the *Avr*9 insert in the PVX-vector) as a general primer and a mutagenic primer that created the required mutation. The second reaction included the general primer N31 (5′-GAC CCT ATG GGC TGT GTT G-3′, downstream of the *Avr*9 insert in the PVX-vector) and a mutagenic primer carrying the mismatch complementary to the mutagenic primer used in the first PCR reaction. PCR amplifications were carried out with AmpliTaq DNA Polymerase (Perkin Elmer-Cetus, Norwalk, CT), in a Perkin Elmer GeneAmp 9600 thermocycler at 25 to 40 cycles of 30 s at 94°C, 30 s at 40 °C, and 20 s at 72°C. After these PCR reactions, the amplified fragments were isolated and corresponding fragments were mixed and subjected to the overlap extension PCR amplification, using the OX10 and N31 primers. The latter PCR reaction was performed using Ultma DNA Polymerase (Perkin Elmer-Cetus, Norwalk, CT), with PCR conditions identical to the first PCR amplifications. After the two-step mutagenesis procedure, the resulting PCR fragment was digested with *Cla*I, and the *Cla*I fragment was isolated and ligated into the *Cla*I site of the PVX expression vector pTXΔGC3A (Hammond-Kosack et al., 1995). Transformants of *E. coli* DH5α carrying the mutant *Avr*9 gene in the correct orientation in the PVX expression vector were selected by PCR screening, and the nucleotide sequence of the PVX construct was verified by DNA sequencing.

Table 3.3 Codon exchanges made in PVX::*Avr*9, encoding alanine and valine (A09V) substitutions.

Mutant	codon exchange	Mutant	codon exchange
Wild Type	-	L13A	CTT::GCT
Y01A	TAC::GCC	G14A	GGA::GCA
C02A	TGT::GCT	Q15A	CAA::GCA
N03A	AAC::GCC	G17A	GGA::GCA
S04A	AGT::GCT	R18A	AGA::GCA
S05A	TCT::GCT	D20A	GAC::GCC
C06A	TGT::GCT	F21A	TTT::GCT
T07A	ACA::GCA	H22A	CAT::GCT
R08A	AGA::GCA	K23A	AAG::GCG
A09V	GCT::GTT	L24A	CTC::GCC
F10A	TTT::GCT	Q25A	CAA::GCA
D11A	GAC::GCC	V27A	GTA::GCA
C12A	TGT::GCT	H28A	CAT::GCT

Table 3.4 Codon exchanges made in PVX::*Avr*9, encoding non-alanine substitutions.

Mutant	codon exchange(s)	Mutant	Codon exchange(s)
Wild Type	-	D20N	GAC::AAC
S04A + G17E	AGT::GCT + GGA::GAA	F21S	TTT::TCT
R08S	AGA::TCA	H22L	CAT::CTT
R08K	AGA::AAA	K23Q	AAG::CAG
F10S	TTT::TCT	L24S	CTC::TCC
D11N	GAC::AAC	Q25E	CAA::GAA
Q15A + R18S	CAA::GCA + AGA::AGC	Q25E + V27I	CAA::GAA + GTA::ATA
L13A + C16stop	CTT::GCT + TGT::TGA	C26G	TGT::GGT
R18K	AGA::AAA	H28L	CAT::CTT

In vitro transcriptions and plant inoculations

For *in vitro* transcription, 1 µg of *Sst*I-linearized PVX plasmid DNA was prepared and used in the mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, Austin, TX). Infectious 5'-capped mRNA was produced according to the manufacturer's instructions. The reaction mix (10 µL) was mixed with 10 µl H_2O, containing 5 µg of bentonite per µL and 20 µL of 50 mM sodium phosphate buffer, pH 7.0, was added. Leaves of 4-week-old *N. clevelandii* plants (four leaves per plant) were inoculated with 10 µl of the mRNA sample. Ten to fourteen days after inoculation, leaves showing systemic mosaic symptoms were harvested for sap preparation. Sap was made by grinding the leaves in a mortar with sand (1 g fresh weight/mL of buffer, 50 mM sodium phosphate, pH 7.0) and stored at -20°C. Three to four-week-old tomato plants were inoculated with 10 µl of sap on both cotyledons and on the top

leaflets of the primary and secondary compound leaf. Plants were grown in a day-night regime of 16 h, 25°C light and 8 h, 20°C dark at 60 to 80 % relative humidity. All inoculations were performed at least three times.

Purification of mutant AVR9 peptides and injections

Several AVR9 peptides were purified from apoplastic fluid (AF) of *N. clevelandii* leaves, inoculated with sap containing the mutant PVX::*Avr*9 construct of choice. For purification of a mutant AVR9 peptide, 25 5-week-old *N. clevelandii* plants were inoculated with sap of leaves from the PVX-infected plants. After 2 weeks, AF was isolated from leaves showing mosaic symptoms, following the method described by De Wit and Spikman (1982). Samples were incubated at 90°C for 10 min to inactivate PVX and to denature heat-labile proteins. High molecular weight proteins in the AF were precipitated by adding acetone to a final concentration of 50% (vol/vol) and overnight incubation at -20°C. After centrifugation, the supernatant containing the heat-stable mutant AVR9 peptide was collected and acetone was removed in a rotary evaporator at 50°C. The mutant peptides were purified further by a combination of cation exchange chromatography on a CM-Sephadex C-25 column (Pharmacia Biotech AB, Uppsala, Sweden), followed by reversed-phase HPLC on a SuperPac Pep-S column (Pharmacia Biotech AB), essentially as described by Van den Ackerveken et al. (1993). The peptides were quantified by comparing the intensity of the AVR9 bands on silver-stained low pH polyacrylamide gels with a defined quantity of AVR9 isolated from *C. fulvum*-infected leaves as a standard (Reisfeld et al., 1962).

Dilutions series of the purified peptides were prepared, and 20 µL of each concentration was injected into leaflets of MM-Cf9 near the main vein. Control injections were performed in leaflets of MM-Cf0. All injections were performed twice.

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CHAPTER 4

CORRELATION BETWEEN BINDING AFFINITY AND NECROSIS-INDUCING ACTIVITY OF MUTANT AVR9 PEPTIDE ELICITORS

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Abstract

The race-specific peptide elicitor AVR9 of the fungus *Cladosporium fulvum* induces a hypersensitive response (HR) only in tomato plants carrying the complementary resistance gene *Cf-9* (MM-Cf9). A binding site for AVR9 is present on plasma membranes of both resistant and susceptible tomato genotypes. We used mutant AVR9 peptides to determine the correlation between elicitor activity of these peptides and their affinity to the binding site in membranes of tomato. Mutant AVR9 peptides were purified from *Nicotiana clevelandii,* inoculated with recombinant potato virus X (PVX) expressing the corresponding *Avr*9 derivative (chapter 3). In addition, several AVR9 peptides were synthesized chemically. Electrospray Mass Spectrometry, Circular Dichroism-, and ^{1}H-NMR-spectroscopy revealed that the peptides were correctly folded. Most AVR9 peptides purified from PVX::*Avr*9–infected *N. clevelandii* contain a single N-acetylglucosamine. The glycosylated AVR9 peptides show lower affinity to the binding site than the non-glycosylated AVR9 peptides, whereas their necrosis-inducing activity was hardly changed. For both the non-glycosylated and glycosylated mutant AVR9 peptides, a positive correlation between their affinity to the membrane-localized binding site and their necrosis-inducing activity in MM-Cf9 tomato was found. This suggests that the AVR9 binding site is required for the induction of HR in MM-Cf9 plants. The perception of AVR9 in resistant and susceptible plants is discussed.

Introduction

The outcome of many plant-pathogen relationships is governed by the presence or absence of matching pathogen avirulence (*Avr*)-genes and plant resistance (*R*)-genes. When both the *Avr*-gene and the matching *R*-gene are expressed, a resistance response is induced. This phenomenon has been described as the gene-for-gene interaction (Flor, 1971). To date, a variety of *Avr*- and *R*-genes have been cloned. The cloned *Avr*-genes include more than 30 bacterial genes (reviewed by Dangl, 1994; Leach and White, 1996), but also viral and fungal genes (Taraporewala and Culver, 1996; Padgett et al., 1997; Rohe et al., 1995; Van Kan et al., 1991; Joosten et al., 1994). Although several *R*-genes have been cloned (Staskawicz et al., 1995), only a few *Avr*-genes with matching specificity have been isolated (presented in Table 4.1). It has been proposed that race-specific resistance results from the direct interaction of the products of an *Avr*-gene and the corresponding *R*-gene (Gabriel and Rolfe, 1990; Staskawicz et al., 1995). At present, evidence supporting this hypothesis is only available for the interaction between one bacterial *Avr*-gene product, AvrPto, and its complementary *R*-gene product, Pto (Tang et al., 1996; Scofield et al., 1996).

The interaction between tomato and the fungal pathogen *Cladosporium fulvum* complies with the gene-for-gene relationship. The tomato *R*-genes *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* confer resistance to races of *C. fulvum* expressing the corresponding *Avr*-genes, *Avr*2, *Avr*4, *Avr*5 and *Avr*9, respectively. The sequences of these *R*-genes predict that they encode extracytoplasmic glycoproteins containing imperfect, 24—amino acid leucine-rich repeat motifs (LRRs), motifs involved in protein-protein interactions (Jones and Jones, 1996; Kobe and Deisenhofer, 1995). The *Avr*4 and *Avr*9 gene have been cloned (Joosten et al., 1994; Van Kan et al., 1991; Van den Ackerveken et al., 1992). The *Avr*4 gene encodes a pre-proprotein of 135 amino acid residues. The mature AVR4 peptide elicitor, of which almost no structure-activity data are available, consists of 86 amino acids, of which 8 are cysteines potentially forming 4 disulfide bonds (Joosten et al., 1997b). The *Avr*9 gene encodes a 63—amino acid pre-proprotein containing one potential glycosylation site (residues N03, S04, S05 of the mature peptide) (Van den Ackerveken et al., 1993). The AVR9 elicitor predominantly present in *C. fulvum*—infected tomato plants, contains 28 amino acids and is not glycosylated (Van den Ackerveken et al., 1993). A variety of larger AVR9 peptides can be found in *in vitro*—grown cultures of

transgenic *C. fulvum* overexpressing the AVR9 elicitor (Van den Ackerveken et al., 1993). Peptides of 28 up to 34 amino acids have been identified, some of which are N-glycosylated, containing two N-acetylglucosamines and a variable number of mannose residues (P.J.G.M. de Wit and P. Vossen, manuscript in preparation). The global fold of the AVR9 peptide has been determined by two-dimensional ^{1}H-NMR (2D-NMR) spectroscopy (Vervoort et al., 1997). The AVR9 elicitor has 3 antiparallel β-sheets with 3 disulfide bonds arranged in a cystine knot (Isaacs, 1995). The AVR9 elicitor is structurally related to small inhibitor peptides, such as protease inhibitors and ion channel blockers (Vervoort et al., 1997).

A high-affinity binding site for the race-specific elicitor AVR9 is present on plasma membranes of tomato genotypes that are either susceptible (MM-Cf0) or resistant (MM-Cf9) to AVR9—producing races of *C. fulvum* (chapter 2). The affinity of AVR9 to the binding site is similar for MM-Cf0 and MM-Cf9 tomato with a dissociation constant (K_d) of 70 pM. The binding site in tomato is specific for the AVR9 peptide, since it does not bind AVR4 or other small cysteine-rich peptides (chapter 2). It is yet unknown whether the high-affinity binding site is required for the AVR9—induced hypersensitive response (HR) in MM-Cf9 tomato plants.

Recently, we have assigned amino acid residues of the AVR9 peptide that are important for necrosis-inducing activity in MM-Cf9 tomato plants, by independently substituting each amino acid of AVR9 by alanine or another amino acid (chapter 3). Elicitor activity of mutant AVR9 peptides was studied by expressing the corresponding mutated *Avr9* gene in MM-Cf9 tomato plants using the potato virus X (PVX) expression system (Chapman et al., 1992). The severity of necrosis induced by each PVX::*Avr9* construct was subsequently assessed. This allowed direct identification of amino acid residues of AVR9 that are important for necrosis-inducing activity. We identified amino acid substitutions resulting in AVR9 mutants with higher, similar or lower necrosis-inducing activity compared to the wild-type AVR9 peptide. Some AVR9 mutants showed no necrosis-inducing activity (chapter 3). The objective of this study was to determine whether the necrosis-inducing activity of mutant AVR9 peptides correlates with their affinity to the tomato binding site, suggesting a key role for the binding site in the induction of AVR9/CF-9-dependent resistance. A selection of AVR9 peptides was either purified from PVX::Avr9—infected *Nicotiana clevelandii*, or synthesized chemically (E. Mahé, P. Vossen, H.W. van den Hooven, D. Le-Nguyen, J.J.M. Vervoort, and P.J.G.M. de Wit; manuscript in preparation). The mutant AVR9 peptides were tested for their affinity to the tomato binding site and for their

Table 4.1 'Matching pairs' of cloned *R*-genes and *Avr* genes

Cloned *R*-gene	Isolated from	Matching *Avr*-gene	Pathogen	Reference
Pto	Tomato	*Avr*Pto	*Pseudomonas syringae* pv *tomato*	Martin et al., 1993
RPS2	Arabidopsis	*Avr*Rpt2	*Pseudomonas syringae* pv *tomato*	Bent et al., 1994
RPM1/RPS3	Arabidopsis	*Avr*Rpm1/*Avr*B	*Pseudomonas syringae* pv *maculicola*	Grant et al., 1995/ Bisgrove et al., 1994
Cf-4	Tomato	*Avr*4	*Cladosporium fulvum*	In: Jones and Jones, 1996
Cf-9	Tomato	*Avr*9	*Cladosporium fulvum*	Jones et al., 1994

necrosis-inducing activity in MM-Cf9 tomato. We show a positive correlation between binding affinity and necrosis-inducing activity for most of the mutant AVR9 peptides. The role of the AVR9 binding site in *Cf-9*-induced resistance is discussed.

Results

All mutant AVR9 peptides are folded correctly

To test whether the various mutant AVR9 peptides are folded correctly, they were analyzed by NMR- and CD-spectroscopy. One to two μg of each peptide was isolated per infected *N. clevelandii* plant, resulting in a total amount of 25 to 50 μg of peptide. One-dimensional ^{1}H-NMR (1D-NMR) spectroscopy of these mutant AVR9 peptides showed characteristics also observed in the spectra of wild-type AVR9, indicating that there are no major structural differences between the peptides. CD-spectra of the peptides showed typical β-sheet characteristics (data not shown).

In addition, milligram quantities of wild-type AVR9 and the mutant AVR9 peptides R08K, F10A, and F21A were chemically synthesized and the peptides were folded (Mahé et al., manuscript in preparation). The conformation of the synthetic AVR9 peptides were studied by 2D-NMR spectroscopy. The synthetic 28—residue AVR9 peptide adopts a similar conformation as the corresponding residues of the 33—residue AVR9 peptide isolated from *in vitro*—grown cultures of *C. fulvum* (H.W. van den Hooven and J.J.M. Vervoort, unpublished data). The mutants R08K and F10A

displayed almost identical chemical shifts, $^{3}J_{NH\text{-}H_{\alpha}}$-coupling constants and NOE data as the synthetic wild-type AVR9, apart from the ring-current shift effects for the F10A mutant (Mahé et al., manuscript in preparation). Thus, the amino acid substitutions of R08K and F10A have little or no effect on the spatial structure of the molecule. The F21A is the only mutant in which significant differences in chemical shifts and $^{3}J_{NH\text{-}H_{\alpha}}$-coupling constants were observed for the protons of residues D20, A21, H22, and K23. However, the conformation of the F21A peptide outside the mutated area showed no significant changes compared to the wild-type AVR9 (Mahé et al., manuscript in preparation). The chemical shift indices (Wishart et al., 1992), which are indicative of secondary structure in proteins, were virtually identical for all four synthetic AVR9 peptides. Thus, the global fold of the synthetic AVR9 peptides is almost identical and the differences in necrosis-inducing activity reflect only the local effect of the amino acid substitution.

AVR9 produced by tomato and tobacco plants is glycosylated

The molecular weights of all mutant AVR9 peptides were determined by electrospray mass spectrometry (ES-MS) (Table 4.2). The wild-type AVR9 peptide elicitor, isolated from *C. fulvum*—infected tomato leaves, and the synthetic wild-type AVR9 have experimental molecular masses of 3188.5 and 3189.1 Dalton (Da), respectively. This is in good agreement with the theoretical molecular mass of 3189.6 Da for the 28—residue peptide containing three disulfide bonds. The chemically synthesized and folded peptides all show the expected mass, indicating the presence of three disulfide bonds. However, the wild-type AVR9 peptide isolated and purified from PVX::*Avr*9—infected *N. clevelandii* shows an experimental mass of 3391.1 Da, which is about 202 Da higher than expected. AVR9 has one potential glycosylation site (N03, S04, S05) and the observed mass difference suggests that the peptide contains one additional N-acetyl-hexosamine residue. The S05A peptide has a mutation in this glycosylation site and shows a calculated mass of 3171.5 Da, which is close to the expected molecular mass of the non-glycosylated peptide with three disulfide bonds (3173.6 Da). Except for the S05A mutant, all other mutant AVR9 peptides, purified from PVX::A*vr*9—infected *N. clevelandii*, show a molecular mass that is 200.9 to 204.0 Da higher than the expected mass (Table 4.2). Thus, the observed mass difference between the experimental and theoretical masses of the AVR9 peptides isolated from PVX::*Avr*9—infected *N. clevelandii* is indeed likely to be due to glycosylation. Since, the glycosylation site in AVR9 predicts N-glycosylation, most probably an N-acetylglucosamine (GlcNac) (Vliegenthart and Montreuil, 1995) is attached to the

asparagine (N03) of these mutant peptides. When the effect of the glycosylation is taken into account, the experimental masses of all mutant AVR9 peptides isolated from PVX::*Avr*9—infected *N. clevelandii* are consistent with their theoretical masses, confirming the altered amino acid sequence and the presence of three disulfide bridges. Most of the glycosylated AVR9 peptides also show one additional smaller peak in the mass spectrum, representing the non-glycosylated form of the peptide (<5% of the primary peak). Only for F10S the peak height of the glycosylated peptide is about twice the height of the non-glycosylated peptide (Table 4.2).

To determine whether, besides *N. clevelandii*, tomato can also glycosylate AVR9, we performed ES-MS experiments on wild-type AVR9, isolated from tomato plants transgenic for the *Avr*9 gene (Honeé, unpublished data). The mass spectrum of this AVR9 showed two peaks with comparable intensities, representing the non-glycosylated peptide (3189.8 Da) and AVR9 with an additional GlcNac residue (3390.5 Da). This shows that both glycosylated and non-glycosylated AVR9 peptides can be produced in plants.

As shown in Figure 4.1A, the glycosylated and non-glycosylated AVR9 peptides had different mobilities on native LpH-PAGE. The non-glycosylated wild-type AVR9, isolated from *C. fulvum*—infected tomato (Figure 4.1, arrow I), migrated slightly faster compared to glycosylated wild-type AVR9 isolated from PVX::*Avr*9—infected *N. clevelandii* (Figure 4.1, arrow II). The migration pattern of the non-glycosylated S05A (*NC*) and the non-glycosylated AVR9 (*WT*) was the same. The other mutant AVR9 peptides, isolated from PVX::*Avr*9—infected *N. clevelandii*, had the same mobility as the glycosylated wild-type AVR9. The two bands of the F10S mutant peptide represent both glycosylated and non-glycosylated forms. The H22L and H28L mutant peptides are less basic and migrate slower on the native LpH-PAGE. Figure 4.1B shows the results of native LpH-PAGE of the synthetic wild-type, R08K, F10A, and F21A peptides. All synthetic AVR9 peptides migrate at the expected position, identical to the control (non-glycosylated) AVR9, isolated from *C. fulvum*—infected tomato. In addition to the major band observed for all peptides, minor, slower migrating bands were observed (Figure 4.1A and B, arrow III). The additional bands, observed for all AVR9 peptides in native LpH-PAGE, are not caused by differences in glycosylation, since they also occur in the lanes containing

Table 4.2 Results of ES-MS analysis of wild-type and mutant peptides AVR9 peptides.

Peptide	Theoretical Mass[a] (Da)	Major peak[b] (Da)	Difference from expected mass[c]	Second peak[d] (Da)	Difference from expected mass
AVR9 *(WT)*[e]	3189.6	3188.5 ± 1.4	1.1		
AVR9 *(SY)*[e]	3189.6	3189.1 ± 0.1	0.5		
AVR9 *(NC)*[e]	3189.6	3391.1 ± 0.7	201.5		
AVR9 *(LE)*[e]	3189.6	3390.5 ± 1.7[f]	200.9	3189.8 ± 0.5[f]	0.2
S05A *(NC)*	3173.6	3171.5 ± 0.3	2.1		
R08K *(SY)*	3161.6	3160.6 ± 0.1	1.0		
R08K *(NC)*	3161.6	3364.2 ± 0.1	202.6	3161.2[g]	0.4
F10A *(SY)*	3113.5	3113.0 ± 0.5	0.5		
F10S *(NC)*	3129.5	3332.3 ± 0.6	202.8	3129.3 ± 0.5[h]	0.2
R18K *(NC)*	3161.6	3364.5 ± 0.1	202.9	3160.6	1.0
F21A *(SY)*	3113.5	3112.6 ± 0.3	0.9		
H22L *(NC)*	3165.6	3369.6 ± 0.4	204.0	3165.3	0.3
L24S *(NC)*	3163.5	3367.4 ± 0.4	203.9		
H28L *(NC)*	3165.6	3368.3 ± 0.6	202.7	3164.8	0.8

[a] The molecular mass of the peptide containing 3 disulfide bonds was calculated.

[b] Mass corresponding to the main peak in the spectrum.

[c] An additional mass of 202 Da can represent the presence of one GlcNac residue.

[d] Mass corresponding to a second peak, which was detected in some of the spectra. The peak height was generally below 5% of the main peak.

[e] AVR9 *(WT)* indicates wild-type AVR9, isolated from *C. fulvum*—infected tomato, *(SY)* indicates chemically synthesized peptide. Peptides isolated from PVX::*Avr*9—infected *N. clevelandii* are designated *(NC)*, and AVR9 *(LE)* is AVR9 isolated from transgenic tomato.

[f] The two peaks showed similar heights.

[g] Very small additional peaks of 3678 and 3686 Da were observed, indicating attachment of larger molecules.

[h] The height of this peak is about half of the height of the main peak.

chemically synthesized AVR9 peptides. These bands could represent slight alterations in the charge of the peptides, possibly resulting from de-amination of glutamine into glutamic acid.

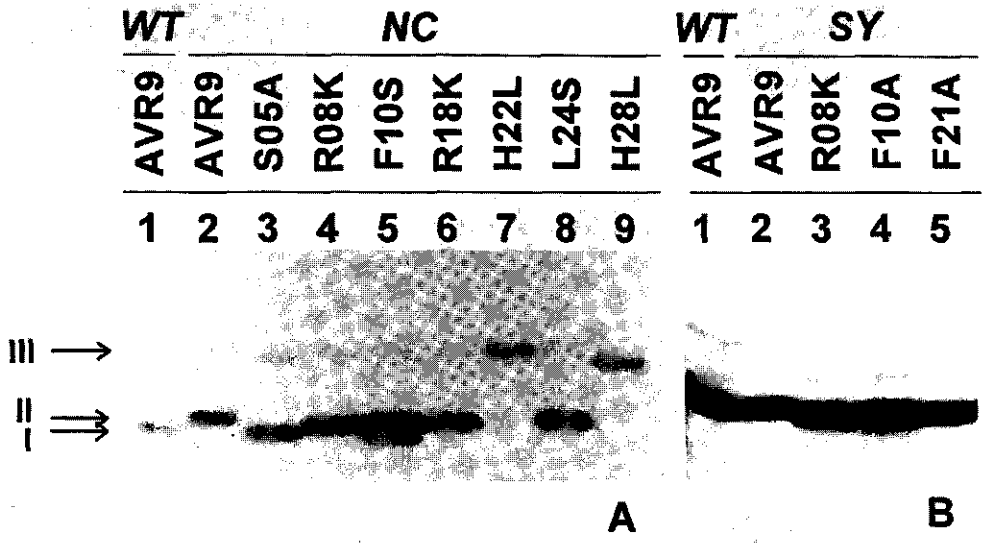

Figure 4.1 **A.** Native LpH-PAGE of wild-type AVR9, isolated from *C. fulvum*–infected tomato *(WT)* and wild-type and mutant AVR9 peptides from PVX::*Avr9*–infected *N. clevelandii (NC)*. Approximately one microgram was loaded per lane. **B.** LpH-PAGE of wild-type *(WT)* and chemically synthesized AVR9 peptides *(SY)*. Approximately two micrograms were loaded per lane.

Mutant AVR9 peptides show different necrosis-inducing activities

The necrosis-inducing activity of the various AVR9 peptides was tested by injecting 20 µl of the peptides with varying concentrations into leaflets of MM-Cf9 tomato plants. The observed necrosis was divided into 4 classes, as presented in Figure 4.2, varying from no necrosis (-) to spreading necrosis (++). Table 4.3 shows a summary of the results of the necrosis-inducing activities of the mutant peptides. The wild-type AVR9 peptide, isolated from *C. fulvum*–infected tomato showed necrosis at concentrations of 0.3 µM and higher (Table 4.3). The glycosylated wild-type AVR9 peptide, isolated from the PVX::*Avr9*–infected *N. clevelandii,* gave similar necrosis-inducing activity as the non-glycosylated wild-type AVR9, indicating that glycosylation does not affect the necrosis-inducing activity. Also, the necrosis-inducing activity of the chemically synthesized wild-type AVR9 peptide was similar compared to the necrosis-inducing activity of wild-type AVR9 from *C. fulvum*–

infected tomato. This is consistent with the 2D-NMR data, showing that the synthetic peptides are correctly folded. The necrosis-inducing activities of the two R08K mutants (synthetic and isolated from PVX::*Avr*9—infected *N. clevelandii*) and of the R18K mutant (isolated from PVX::*Avr*9—infected *N. clevelandii*) were stronger than the necrosis-inducing activity of wild-type AVR9. At 0.1 μM, these mutants induced necrosis, whereas the wild-type AVR9 only induced chlorosis at this concentration. The necrosis-inducing activities of S05A, isolated from the PVX::*Avr*9—infected *N. clevelandii*, and of the synthetic F10A mutant were slightly reduced (Table 4.3). Mutants F10S, H22L and H28L, isolated from PVX::*Avr*9—infected *N. clevelandii*, induced necrosis only at 3 μM and higher. The L24S isolated from PVX::*Avr*9—infected *N. clevelandii* and the synthetic F21A, did not show necrosis at the injected concentrations, but chlorosis was observed at 30 μM and 100 μM for the L24S and F21A mutant, respectively.

There is a clear correlation between the necrosis-inducing activity exhibited by a peptide injected in MM-Cf9 tomato leaflets and the systemic necrosis induced by the corresponding PVX::*Avr*9 derivative in MM-Cf9 plants described previously (chapter 3). Fast and severe necrosis in the PVX-based assay corresponds to strong necrosis-inducing activity of the isolated peptide. Similarly, slow or little necrosis in the PVX-based assay corresponds to low necrosis-inducing activity of the isolated peptide, whereas no necrosis induced by the PVX::*Avr*9 derivative, concurs with no necrosis-inducing activity of the isolated peptide. Thus, the initial selection of peptides based on the PVX::*Avr*9—infected MM-Cf9 provides a reliable indication of the necrosis-inducing activity of the isolated AVR9 peptides.

Mutant AVR9 peptides show different binding affinities

To investigate a possible correlation between the necrosis-inducing activity of the various AVR9 peptides in MM-Cf9 plants and their affinity to the AVR9 binding site in tomato membranes, the dissociation constants (K_ds) of the peptides were determined by competition binding assays. The K_ds of the non-glycosylated peptides are summarized in Table 4.4A. Figure 4.3 shows a scattergram, in which the K_ds of the different AVR9 peptides are plotted against their necrosis-inducing activity. No accurate quantitative assay for the elicitor activity of AVR9 peptides is available and therefore, the minimal concentration of a (mutant) AVR9 peptide

Figure 4.2 Representation of 4 classes of AVR9—induced necrosis in MM-Cf9 leaflets as presented in Table 4.3. Tomato (MM-Cf9) leaflets were injected with 20 µL of AVR9, isolated from *C. fulvum*—infected tomato. From left to right concentrations of 0.03 µM (-, no necrosis), 0.1 µM (±, chlorosis), 0.3 µM (+, necrosis) and 10 µM (++, spreading necrosis) were injected.

required to induce necrosis (Table 4.3) was used to represent its activity. The binding affinity of most of the non-glycosylated AVR9 peptides correlates positively with their necrosis-inducing activity. The synthetic R08K mutant shows higher necrosis-inducing activity than wild-type AVR9 and has higher affinity to the binding site. Non-glycosylated mutants with low (S05A and F10A) and no detectable (F21A) necrosis-inducing activity have slightly lower (S05A) or much lower (F10A and F21A) affinities to the binding site. Non-binding AVR9 mutants have never been observed. Carboxypeptidase inhibitor and ω-conotoxin, two cystine knotted peptides structurally homologous to AVR9, were included as non-binding controls (Sevilla et al., 1993; Chang et al., 1994). These do not compete for AVR9 binding, even at concentrations as high as 10 µM.

Table 4.3 Necrosis observed upon injection with different concentrations of wild-type and mutant AVR9 peptides. Peptide designations are as in Table 4.2. Examples of leaves showing no necrosis (-), chlorosis (±), necrosis (+) and spreading necrosis (++) are presented in Figure 4.3. The highest concentration tested (100 μM for all synthetic peptides and wild-type AVR9 and 1 to 10 μM for all other peptides), was also tested in MM-Cf0 leaflets. No necrosis or chlorosis was observed in MM-Cf0 for any of the peptides.

Mutant	Conc. (μM)	0.03	0.1	0.3	1	3	10	30	100
AVR9 *(NC)*		-	±	+	+	++	++	NT	NT
AVR9 *(WT)*		-	±	+	+	++	++	++	++
AVR9 *(SY)*		-	-	+	+	++	++	++	++
S05A *(NC)*		NT	-	-	+	+	NT	NT	NT
R08K *(NC)*		-	+	+	++	NT	NT	NT	NT
R08K *(SY)*		-	+	+	++	++	++	++	++
F10A *(SY)*		-	-	±	+	+	++	++	++
F10S *(NC)*		NT	NT	-	±	+	++	NT	NT
R18K *(NC)*		-	+	+	+	NT	NT	NT	NT
F21A *(SY)*		-	-	-	-	-	-	-	±
H22L *(NC)*		NT	NT	-	±	+	++	NT	NT
L24S *(NC)*		NT	NT	-	-	-	-	±	NT
H28L *(NC)*		NT	NT	-	-	+	++	NT	NT

Table 4.4B shows the K_d of the glycosylated AVR9 peptides, isolated from PVX::*Avr9*–infected *N. clevelandii*. A positive correlation between binding affinity and necrosis-inducing activity is also observed for the glycosylated peptides. Comparison of both glycosylated R08K and R18K mutants with the glycosylated wild-type AVR9 shows a higher affinity of both mutant peptides, and an increased necrosis-inducing activity. The glycosylated mutant peptides, F10S, H22L, and H28L, show a lower affinity than the glycosylated wild-type AVR9, and a lower necrosis-inducing activity. The lowest affinity is observed for the inactive L24S mutant. The affinities of the glycosylated peptides are approximately 10-50 fold lower than the affinities of the non-glycosylated peptides.

Table 4.4 Affinities of AVR9 peptides as determined by competition binding analyses. AVR9 (*WT*) indicates wild-type AVR9, isolated from *C. fulvum*—infected tomato, (*SY*) indicates synthetic AVR9 peptides. Peptides isolated from PVX::*Avr9*—infected *N. clevelandii* are designated (*NC*). n indicates the number of experiments.

Table 4.4A Non-glycosylated peptides

Peptide	K_d (nM)	n
AVR9 *(WT)*	0.41 ± 0.51	16
AVR9 *(SY)*	0.40 ± 0.33	2
S05A *(NC)*[a]	1.37	1
R08K *(SY)*	0.18 ± 0.19	2
F10A *(SY)*	7.72 ± 5.47	3
F21A *(SY)*	12.5 ± 3.6	5
CPI[b]	no comp. (> 10000)	1
CTX-GVIA[b]	no comp. (> 10000)	1

Table 4.4B Glycosylated peptides

Peptide	K_d (nM)	n
AVR9 *(NC)*	9.76 ± 7.15	3
R08K *(NC)*	7.55 ± 1.40	3
F10S *(NC)*[c]	57.7± 36.3	2
R18K *(NC)*	3.74 ± 0.71	2
H22L *(NC)*	70.8 ± 6.45	2
L24S *(NC)*[d]	127 ± 55.0	2
L24S *(NC)*[d]	98.1 ± 28.1	2
H28L *(NC)*[a]	37.4	1

[a] For S05A (*NC*) and H28L (*NC*) only one experiment could be performed, due to the limited quantities of these peptides available.

[b] Carboxypeptidase inhibitor (CPI) and ω-conotoxin GVIA (CTX-GVIA) are two peptides with structural homology to AVR9 (Isaacs, 1995).

[c] Mixture of glycosylated and non-glycosylated peptides

[d] Results of two independent isolations from *N. clevelandii*.

All competition binding assays were also performed using membranes of the susceptible tomato genotype MM-Cf0. The same differences in affinity were observed with membranes of MM-Cf0 plants as compared to membranes of MM-Cf9 plants, indicating that the high-affinity binding sites in resistant and susceptible plants have identical binding properties. Also, in competition binding experiments to tobacco membranes (cv Petit Havana), which also have a high-affinity binding site for AVR9, the chemically synthesized AVR9 peptides showed values similar to those obtained for tomato membranes (results not shown).

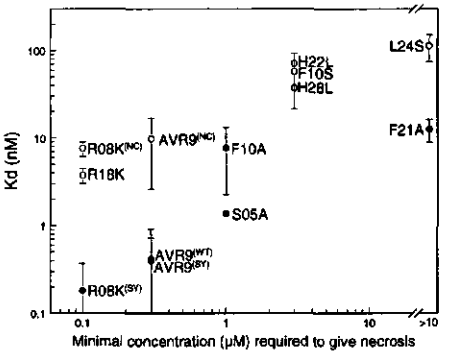

Figure 4.3 Scattergram of the correlation between dissociation constants (*Kds*) and necrosis-inducing activity. The K_ds are as shown in Table 4.4. The necrosis-inducing activity is represented by the minimal concentration required to induce necrotic lesions in MM-Cf9 tomato (in Table 4.3). Non-glycosylated peptides are represented by closed circles and include all chemically synthesized peptides, AVR9 *(WT)*, isolated from *C. fulvum*—infected tomato and the glycosylation mutant S05A, isolated from PVX::*Avr9*—infected *N. clevelandii*. The glycosylated peptides are represented by open circles and are all isolated from PVX::*Avr9*—infected *N. clevelandii*. Name designations (*WT, SY* and *NC*), are as in legend to Figure 4.2. Only for the wild-type AVR9 and for R08K, these designations are shown in the graph, since more than one form of these peptides is presented.

Discussion

Correlation between necrosis-inducing activity and binding affinity of AVR9 peptides

A high-affinity binding site for AVR9 is present in plasma membrane fractions of MM-Cf0 and MM-Cf9 tomato genotypes (chapter 2). Our working hypothesis predicted that the high-affinity binding site for AVR9 is required for *Cf-9*—dependent HR. We used mutant AVR9 peptides to investigate a possible correlation between the affinity of these peptides to the binding site and their necrosis-inducing activity. Mutant AVR9 peptides were produced by expression of PVX::*Avr9* derivatives in *N. clevelandii* plants and by chemical synthesis. The mutant AVR9 peptides were correctly folded and therefore, their necrosis-inducing activities and binding

affinities are expected to reflect only the local effect of the amino acid substitution. AVR9 peptides produced by expression of PVX::*Avr*9 in *N. clevelandii* are mostly glycosylated and contain one additional GlcNac residue. Figure 4.3 shows that there is a positive correlation between necrosis-inducing activity of AVR9 peptides in MM-Cf9 plants and their affinity to the binding site, for either the non-glycosylated or the glycosylated peptides. This suggests that the high affinity binding site in MM-Cf9 is indeed required for *Cf-9*–dependent resistance. In MM-Cf0 tomato plants, which also have a high-affinity binding site, necrosis is not induced, suggesting that in MM-Cf9 an additional factor is involved in initiating the signal cascade that results in HR. Although we found a positive correlation between binding affinity and necrosis-inducing activity for most AVR9 peptides, this did not hold for all peptides. The glycosylated and the non-glycosylated AVR9 peptides have similar necrosis-inducing activities, whereas their binding affinities show a large difference. This will be discussed in more detail in the paragraph '*glycosylation of AVR9*'. Also, the F21A and L24S mutants have no necrosis-inducing activity, but still showed low affinity to the binding site. This is discussed in the paragraph '*inactive AVR9 peptides*'.

Glycosylation of AVR9

In this study, we have shown that glycosylation of AVR9 can occur in plants. AVR9 peptides with various degrees of glycosylation have also been found in culture filtrates of transgenic *C. fulvum* strains, overexpressing the *Avr*9 gene (P.J.G.M. de Wit and P. Vossen, manuscript in preparation). Thus, AVR9 can be glycosylated by different organisms. Usually, N-glycosylation in eukaryotes involves the attachment of two N-acetylglucosamines and a number of mannose residues (Vliegenthart and Montreuil, 1995). We have shown by mass spectrometry that most of the wild-type AVR9 peptide, produced in PVX::*Avr*9–infected *N. clevelandii* plants, contained one GlcNac residue, whereas approximately half of the AVR9, isolated from *Avr*9-transgenic tomato, contained one GlcNac residue. The presence of non-glycosylated AVR9 peptides and the unusual attachment of only one GlcNac residue, suggest that deglycosylation of AVR9 by glycosidases occurs in the apoplast. Deglycosylation of foreign peptides is a general phenomenon in plants (Cervone et al., 1989).

Although the necrosis-inducing activities of non-glycosylated and glycosylated AVR9 peptides are similar, they show a large difference in binding affinity. Non-glycosylated and glycosylated wild-type AVR9 (Table 4.4A and B) show a 24-fold

difference in affinity, while non-glycosylated R08K and glycosylated R08K (Table 4.4A and B) show a 42-fold difference in affinity. As discussed above, AVR9 may be de-glycosylated in the apoplast of tomato. This would explain (at least part of) the observed discrepancy between necrosis-inducing activity and affinity, since circa half of the AVR9 molecules isolated from tomato are potentially de-glycosylated. In addition, the observed difference in binding affinity between glycosylated and non-glycosylated peptides could be due to either a higher solubility of glycosylated peptides in the *in vivo* assays, or a lower solubility in the *in vitro* assays, where the solubilized membranes contribute to a hydrophobic environment. For the cystine-knotted peptide ω-conotoxin, which has high structural homology to AVR9, structure-function studies showed a poor correlation between binding to the N-type calcium channel and the channel-blocking activity (Lew et al., 1997). The authors hypothesized that this could be due to an altered conformation of the calcium channel in the *in vitro* assays. This might also (partly) explain the discrepancies between binding and activity of some of our mutant AVR9 peptides.

Inactive AVR9 peptides

Not only the glycosylated versus the non-glycosylated peptides show a discrepancy between binding affinity and necrosis-inducing activity. The F21A and L24S mutants did not induce necrosis upon injection into MM-Cf9 leaflets up to concentrations of 100 and 30 μM, respectively, but still showed low affinity to the binding site. Both peptides bound to the AVR9 binding site with slightly lower affinity than non-glycosylated (for F21A) or glycosylated (for L24S) AVR9 mutants that exhibited low necrosis-inducing activity. Possibly, the inactive peptides function as antagonists of AVR9. No accurate quantitative *in vitro* assay for determining the activity of AVR9 peptides is available, which could demonstrate whether inactive AVR9 peptides block the responses induced by active AVR9. Alternative explanations could be that the *in vivo* binding conditions may not be optimal for F21A and L24S, their affinity may be too low to induce necrosis, or they may be degraded by tomato proteases before reaching the binding site. The latter suggestion might apply for the F21A peptide, which has similar affinity to the binding site as the F10A, but no necrosis-inducing activity.

Models for the role of the AVR9 binding site in necrosis induction.

Based on the positive correlation between binding affinity and necrosis-inducing activity of AVR9 peptides (found in a certain range of concentrations), we postulate

that the high-affinity binding site for AVR9 is required to initiate the resistance response in tomato plants carrying the *Cf-9* resistance gene. We present two models to discuss the role of the binding site in initiating the AVR9-CF-9–dependent HR.

The initial model for the recognition of elicitors by resistant plants has been the elicitor-receptor model, which assumed that R-genes encode the receptors for the Avr-gene product (Gabriel and Rolfe, 1990). This model is unlikely for the AVR9–CF-9 interaction, since we have shown that binding of AVR9 is not restricted to tomato plants carrying the *Cf-9* resistance gene (chapter 2) and the high-affinity binding site for AVR9 is not encoded by the *Cf-9* resistance gene, or a gene homologous to *Cf-9* (chapter 5). As yet, the elicitor-receptor model has only been verified for the interaction between tomato and the bacterial pathogen *Pseudomonas syringae* pv tomato, where the resistance gene product, Pto kinase, and the avirulence gene product, AvrPto, were shown to interact physically (Tang et al., 1996; Scofield et al., 1996). It has been proposed that AvrPto mediates the interaction between the Pto kinase and the LRR-containing protein Prf, hereby activating the signal cascade. Similarly, binding of AVR9 could mediate the interaction between the AVR9 binding protein and the LRR protein CF-9. This is schematically represented in Figure 4.4A. Possibly, binding induces recruitment of CF-9 into the binding site-AVR9 complex. The resulting CF-9-AVR9-binding-site–complex will subsequently initiate the resistance signal cascade. This model is supported by the correlation between necrosis-inducing activity of several AVR9 peptides and their affinity to the high-affinity binding site, indicating that this binding site is required for the induction of the resistance response. However, at the moment, we cannot fully exclude the possibility that CF-9 directly binds AVR9 with low affinity. This alternative model is schematically represented in Figure 4.4B. Given the observed correlation between affinity and activity of AVR9 peptides, this second model would imply that the amino acids of AVR9 required for binding to the binding site, are similar to those required for binding to CF-9. The preferred model to explain the data described in this paper is, however, the model presented in Figure 4.4A, since an AVR9 low-affinity binding site has never been detected in MM-Cf9 membranes (chapter 2). Isolation and characterization of the high-affinity binding site and cloning of the encoding gene will help to unravel the first step in the process of HR induction in *Cf-9* resistant plants.

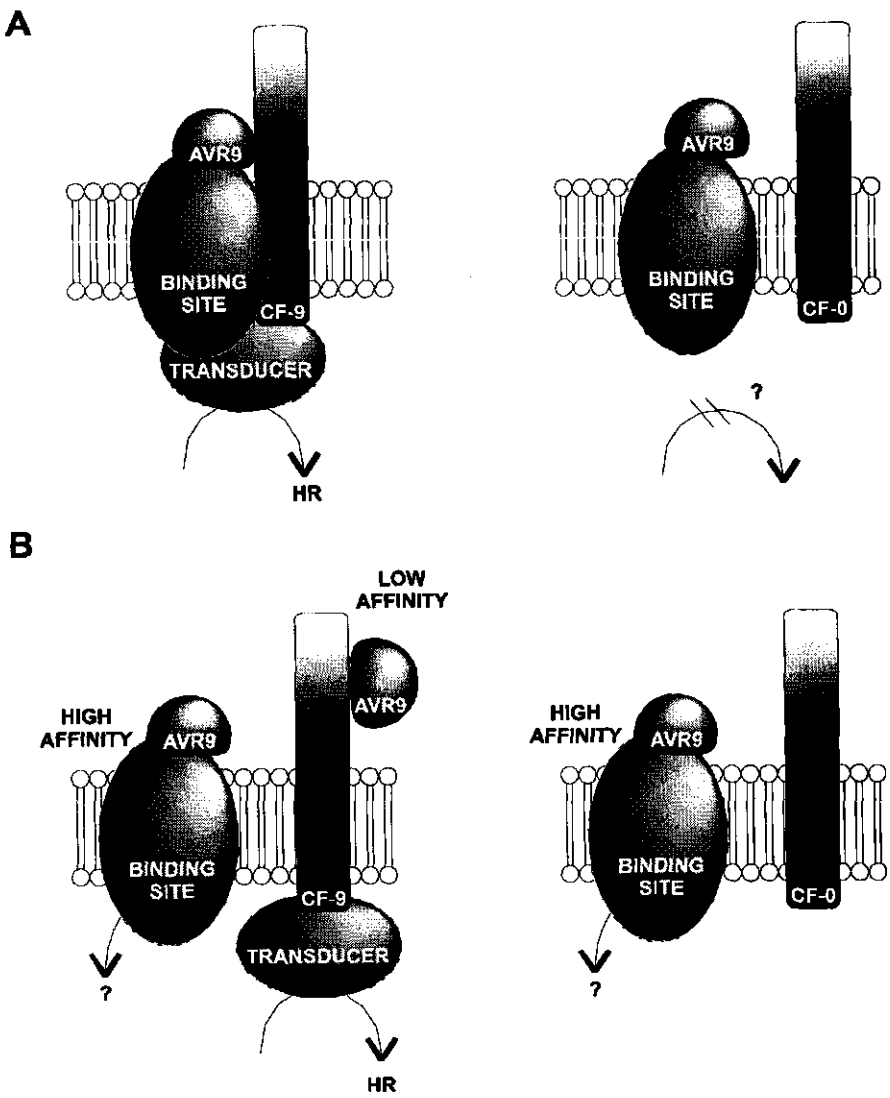

Figure 4.4 Two models of the perception of AVR9 by resistant (left, *Cf-9*) and susceptible (right, *Cf-0*) tomato genotypes. **A.** Model where AVR9 binding mediates the interaction between CF-9 and the binding-site—AVR9 complex. **B.** Alternative model, where the high-affinity binding site is not required to initiate HR, but HR is induced by low-affinity binding of AVR9 to CF-9.

Material and methods

Isolation of (mutant) AVR9 peptides from PVX::Avr9–infected plants

The necrosis-inducing activity of mutant AVR9 peptides had been assessed previously by determining systemic necrosis induced by mutant PVX::*Avr9* derivatives on MM-Cf9 tomato plants (chapter 3). Mutant AVR9 peptides with higher (R08K, R18K), lower (S05A, F10A, F10S, H22L, H28L) or no detectable (F21A, L24S) necrosis-inducing activity in the PVX-based assay were selected for further studies. Several of these mutant peptides, including S05A, R08K, F10S, R18K, H22L, L24S, H28L, and wild-type AVR9 were isolated from apoplastic fluid (AF) of *N. clevelandii*, inoculated with the corresponding PVX::*Avr9* derivative. Mutant PVX::*Avr9* constructs were made as described previously (chapter 3). Plasmid DNA was isolated and infectious 5'-capped mRNA was obtained using the mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, Austin, Texas). Leaves of four-week old *N. clevelandii* plants were inoculated with the infectious mRNA as described (chapter 3). Ten to 14 days after inoculation, leaves showing systemic mosaic symptoms were harvested for preparation of sap containing the infectious virus. For the purification of each mutant AVR9 peptide, at least 25 five-week old *N. clevelandii* plants were inoculated with sap containing the corresponding infectious virus. After two weeks, leaves that showed mosaic symptoms were selected and apoplastic fluid (AF) was isolated (De Wit and Spikman, 1982). Mutant AVR9 peptides were purified as described previously (chapter 3). The peptides were further characterized by low-pH polyacrylamide gel electrophoresis (LpH-PAGE), electrospray mass spectrometry (ES-MS), one-dimensional ^{1}H-NMR (1D-NMR) spectroscopy, and Circular Dichroism (CD) spectroscopy.

In addition, AVR9 was isolated from AF of transgenic tomato plants expressing the *Avr9* gene under transcriptional control of the constitutive CaMV35S promoter, as described by Honée et al. (1995). AVR9 was also isolated from AF of *C. fulvum*–infected tomato, as described by Van den Ackerveken et al. (1993).

Chemical synthesis of AVR9 peptides

Wild-type AVR9 and mutant peptides with higher (R08K), lower (F10A) and no necrosis-inducing activity (F21A) were chemically synthesized and folded (Mahé et al., manuscript in preparation). These peptides were purified on reversed-phase HPLC and were analyzed by LpH-PAGE, ES-MS, and 2-dimensional ^{1}H-NMR (2D-NMR) spectroscopy.

Quantification of AVR9 peptides.

LpH-PAGE (Reisfeld et al., 1962) was performed to estimate the concentrations of the mutant AVR9 peptides. Different dilutions of the peptides were analyzed on gel and the intensity of both the Coomassie-stained and silver-stained AVR9 peptide bands was visually compared with the intensity of 1 μg of the wild-type AVR9 isolated from *C. fulvum*–infected tomato plants, which was used as standard. The concentration of the standard AVR9 was determined by OD_{280} measurements, using a molar extinction coefficient of 1640, as determined by the GCG sequence analysis software package.

^{1}H-NMR- and CD-spectroscopy

1D-NMR spectra were recorded as described by Vervoort et al. (1997). CD spectra were recorded on a JASCO DP J-600 Spectropolarimeter connected to an IBM-PC. Quartz cells with 0.1 cm path length were used in the wavelength region from 190 to 260 nm (scan speed 50 nm min^{-1}, time constant 1.0 sec, band width 1.0 nm). Ten scans were averaged and smoothed subsequently.

The global fold and disulfide bridging of the synthetic AVR9 peptides (R08K, F10A and F21A) were investigated by 2D-NMR spectroscopy and were compared to synthetic wild-type AVR9 (Mahé et al., manuscript in preparation). The molecules were dissolved in 0.5 mL H_20:$^2H_20$ (9:1) and the pH of all samples was adjusted to 5.0. Measurements were conducted at 5°C on a Bruker AMX2 600 operating at 600 MHz. 1D-NMR spectra and nuclear Overhauser enhancement spectroscopy (NOESY) (Jeener et al., 1979; Bodenhausen et al., 1984), total correlation spectroscopy (TOCSY) (Bax and Davis, 1985; Griesinger et al., 1988) and double-quantum-filtered correlated spectroscopy (DQF-COSY) (Rance et al., 1983) experiments were recorded. $^3J_{NH\text{-}H_\alpha}$-coupling constants were determined by inverse Fourier transformation (Szyperski et al., 1992).

Mass Spectrometry

ES-MS of all peptides was performed on a Finnigan MAT SSQ-710 machine. Ten μg of purified AVR9 was dissolved in a mixture of methanol/water (80/20, v/v) + 1 % acetic acid and infused with a flow rate of 1 μl min^{-1}. For electrical contact a sheath flow of 1 μL min^{-1} was used. Nitrogen was used as drying gas at a temperature of 250°C. The mass spectra were collected in the profile mode, scanning the MS with 1 s per scan. For each sample 64 scans were averaged. The molecular mass was calculated with the deconvolution program BIOMASS.

Membrane isolations and competition binding

Microsomal membranes were isolated from leaves of tomato MM-Cf0 and MM-Cf9, as described previously (chapter 2). Competition binding assays with mutant AVR9 peptides were performed as described (chapter 2), using a volume of 100 μl per reaction.

Briefly, 10 µg of membrane protein was resuspended in 80 µl binding buffer (10 mM phosphate buffer, pH 6.0, 0.1% BSA) and 10 µl of 10^{-10}M ^{125}I-AVR9 and 10 µl of competitor peptide were added. Binding was performed by incubation at 37 °C for 3 hr under gentle shaking in a water bath. Glass fiber filters (Whatman GF/F) were soaked for 1 to 2 hr in 0.5% polyethylenimine, transferred to a Millipore filtration manifold, and washed with 5 mL of H_2O, and 2 mL of binding buffer. Filtration of the samples was carried out at 10^4 Pa and filters were subsequently washed with 12 mL of binding buffer. The filters were transferred to scintillation vials and 3 mL of LumaSafe Plus (LUMAC.LSC B.V., Groningen, The Netherlands) was added. Radioactivity was counted in a Beckman LS-6000 TA scintillation counter.

Necrosis-inducing activity assays

Peptides were further tested for necrosis-inducing activity by injection assays. Dilution series of the peptides were prepared and 20 µl of each concentration was injected near the main-vein of a MM-Cf9 leaflet (De Wit et al., 1985). Control injections were performed in MM-Cf0 leaflets.

Acknowledgments

Transgenic tomato plant, expressing the *Avr*9 gene, were kindly provided by Maarten Stuiver, MOGEN International NV, Leiden, The Netherlands. Bianca van Haperen performed the binding experiments using tobacco membranes. We thank Rob van der Hoeven (Leiden, The Netherlands) for performing the ES-MS experiments. Renier van der Hoorn assisted in preparing Figure 4.4. Matthieu H.A.J. Joosten and Robert C. Schuurink are acknowledged for critically reading the manuscript. Jacques J.M. Vervoort is acknowledged for helpful discussions.

CHAPTER 5

THE *CF-9* RESISTANCE GENE NOR ITS HOMOLOGUES ARE REQUIRED FOR HIGH-AFFINITY BINDING OF THE AVR9 ELICITOR OF *CLADOSPORIUM FULVUM*

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Abstract

A high-affinity binding site for the AVR9 peptide elicitor of the tomato pathogen *Cladosporium fulvum* is present on plasma membranes of tomato genotypes carrying the *Cf-9* resistance gene, but also on tomato genotypes lacking this gene (chapter 2). These tomato genotypes contain DNA sequences homologous to the *Cf-9* resistance gene (Jones et al., 1994). Here, we studied whether the *Cf-9* resistance gene or its homologues code for an AVR9 binding site. We have tested binding of AVR9 to microsomal membranes of a variety of solanaceous and non-solanaceous plant species and analyzed these species for the presence of *Cf-9*-homologues. We show that a high-affinity binding site for AVR9 is present on membranes of all solanaceous species tested. These species all contain homologues of the *Cf-9* resistance gene. A high affinity binding site for AVR9 is also present on membranes of the non-solanaceous plant species cucumber, barley and oat. These latter species however do not contain homologues of the *Cf-9* resistance gene. Furthermore, membranes of tobacco, transgenic for the *Cf-9* resistance gene, showed no increase in the number of AVR9 binding sites. Arabidopsis does not have a binding site for AVR9 and membranes of Arabidopsis, transgenic for the *Cf-9* resistance gene, also showed no AVR9 binding. We conclude that the *Cf-9* resistance gene or its homologues are not required for high-affinity binding of AVR9.

Introduction

Plants employ a diverse array of defense responses to protect themselves against invading pathogens. These responses include the oxidative burst and hypersensitive cell death (Hammond-Kosack and Jones, 1996), which can be triggered by a variety of elicitors from different pathogens. Plant receptors are thought to play an important role in the recognition of elicitors (Gabriel and Rolfe, 1990). Receptors or high-affinity binding sites for fungal elicitor peptides and oligosaccharides have been identified in soybean, parsley, tomato and tobacco (Cosio et al., 1988; Nürnberger et al., 1994; Basse et al., 1993; chapter 2; Wendehenne et al., 1995). The nature of these elicitor binding sites remains to be resolved, but it is suggested that the binding sites for race-specific elicitors are encoded by the corresponding resistance genes (Hammond-Kosack and Jones, 1997). Resistance genes against a variety of plant pathogens have been cloned (reviewed by De Wit, 1997). Many of these resistance genes encode proteins that contain a leucine-rich repeat (LRR) region, located either cytoplasmic, or extracytoplasmic (De Wit, 1997; Jones and Jones, 1996; Hammond-Kosack et al., 1996). LRR motifs are involved in protein-protein interactions and a single LRR-motif can be sufficient to infer specific binding of a peptide (Kobe and Deisenhofer, 1995; Windisch et al., 1995). This suggests a direct interaction between proteinaceous elicitors and the LRR-containing resistance gene products. The only resistance gene product for which a direct interaction with an avirulence gene product has been demonstrated is the tomato Pto kinase, which mediates recognition of AvrPto from *Pseudomonas syringae* pv. *tomato* (Tang et al., 1996; Scofield et al., 1996). However, Pto kinase is one of the few resistance gene products which does not contain LRRs.

We have used the interaction between the fungal pathogen *Cladosporium fulvum* and its only host, tomato, to characterize the molecular basis of pathogen recognition. The interaction between *C. fulvum* and tomato complies with the gene-for-gene model, where resistance is based on specific recognition of avirulence factors of the fungus by the plant (Flor, 1971). The avirulence genes *Avr*4 and *Avr*9 of *C. fulvum* have been cloned and characterized (Joosten et al., 1994; Van Kan et al., 1991; Van den Ackerveken et al., 1992). *Avr*4 and *Avr*9 encode small cysteine-rich extracellular peptides of 86 and 28 amino acids, respectively (Joosten et al., 1997b; Van den Ackerveken et al., 1993). Tomato plants, which contain the *Cf-4*

(MM-Cf4) or *Cf-9* (MM-Cf9) resistance gene, are resistant to races of *C. fulvum* that produce the AVR4 or AVR9 elicitor, respectively. The resistance genes *Cf-4* and *Cf-9* are predicted to encode very similar extracytoplasmic proteins, containing 25 imperfect LRR motifs for CF-4, and 27 for CF-9 (Jones and Jones, 1996; Jones et al., 1994). The carboxy-terminal parts of the two proteins are identical, indicating that specificity resides in the more variable LRR-region at the N-terminal parts of both proteins (Jones and Jones, 1996). It has been suggested that the proteins encoded by the resistance genes *Cf-4* and *Cf-9* are directly involved in perception of the AVR4 and AVR9 elicitor, respectively (chapter 1; Jones and Jones, 1996). The AVR9 peptide is the first specific, gene-for-gene-based elicitor for which a binding site has been identified (chapter 2). It has been demonstrated that high-affinity binding sites for AVR9 are present on membranes of resistant (MM-Cf9) and susceptible (MM-Cf0) tomato genotypes. Although the AVR9 binding site is not confined to plants that carry the functional *Cf-9* resistance gene, a role for the binding site in the induction of *Cf-9*-dependent resistance is suggested by the apparent correlation between elicitor activity of mutant AVR9 peptides and their affinity to the binding site (chapter 4).

The involvement of partly homologous proteins in initiating a biological response has been demonstrated for several recognition systems (Heldin, 1995; Braun and Walker, 1996). Furthermore, several resistance genes have been revealed by DNA gel blot analyses to reside within a cluster of related gene sequences (Jones et al., 1994; Whitham et al., 1994; Song et al., 1995; Parker et al., 1997). Because the binding site for the AVR9 peptide is present in tomato genotypes with and without a functional *Cf-9* gene, and the *Cf-9* gene belongs to a gene family which is present in these tomato genotypes (Jones et al., 1994), it has been proposed that not only the CF-9 protein itself, but also homologues of CF-9 could be involved in AVR9 binding (Dangl, 1995; De Wit, 1995; chapter 2).

To determine whether the CF-9 protein or CF-9-homologous proteins are involved in binding of AVR9, we have tried to correlate the presence of the high-affinity binding site for AVR9 with the presence of homologues of the *Cf-9* resistance gene. Therefore, DNA gel blot analyses of various solanaceous and non-solanaceous plant species were performed to reveal the presence of genes with homology to the *Cf-9* resistance gene. Membranes from these species were used in AVR9 binding assays and results were compared to the results of the DNA gel blot analyses. Furthermore, the involvement of the CF-9 protein in AVR9 perception was studied

in more detail by performing AVR9 binding experiments with membranes from tobacco and Arabidopsis plants, transgenic for the *Cf-9* resistance gene. We show that untransformed tobacco contains a high-affinity binding site for AVR9. If *Cf-9* or its homologues would encode this binding site, possibly the number of binding sites in the *Cf-9*-transgenic tobacco would change. Arabidopsis does not contain a high-affinity binding site for AVR9 (chapter 2). If the *Cf-9* resistance gene would encode the high-affinity binding site for AVR9, membranes from *Cf-9*-transgenic Arabidopsis plants are expected to show binding of AVR9. In our studies, we found no experimental evidence that the *Cf-9* encoded protein is directly involved in the perception of AVR9.

Results

Membranes of various plant species contain a high-affinity binding site for AVR9

Membranes from the solanaceous species tomato, potato, petunia, Datura, pepper and tobacco and membranes from the non-solanaceous plant species wheat, barley, rice, oat, cucumber, lettuce, carrot, brussels sprouts, and Arabidopsis were tested for specific binding of the ^{125}I-AVR9 elicitor. Microsomal membranes of all solanaceous species tested bind ^{125}I-AVR9. Of the non-solanaceous plant species, only cucumber, barley and oat membranes specifically bind ^{125}I-AVR9. Table 5.1 lists the apparent dissociation constants (K_d values) and receptor concentrations (R_t values) for the plant species that bind AVR9. Values are based on Scatchard analyses of which typical examples of a solanaceous plant (tomato MM-Cf0), a non-solanaceous plant (oat) and a non-binding plant (Arabidopsis) are shown in Figure 5.1. The calculated K_d values vary from 47 pM (for one of the oat samples) to 169 pM (for one of the Datura samples). Although the K_d values of the different plant species vary, they are in the same order of magnitude as the K_d value found for tomato (K_d ~70 pM). The R_t values of the plant species vary between different membrane isolations, but are also in the same range as the R_t observed for tomato.

Table 5.1 K_d values and R_t values of microsomal membranes of plant species that specifically bind AVR9. Membrane isolations and binding experiments were performed in at least two independent experiments, except for cucumber membranes (one isolation). Five microgram of microsomal membrane protein was used per assay, except for the second experiment with tomato, where 20 microgram was used. Each row represents one experiment.

Plant species	K_d (pM)[a]	R_t (pmol per milligram membrane protein)[a]
Tomato (MM-Cf9)	70	0.80
	77	0.33
Tobacco	54	0.65
	60	0.92
Datura	76	0.47
	169	1.98
Petunia	61	0.17
	90	0.62
Potato	54	0.61
	70	0.92
Pepper	97	0.27
	146	0.36
Cucumber	73	0.29
	NT[b]	NT[b]
Barley	124	0.21
	100	0.56
Oat	47	0.88
	130	0.68

[a] K_d and R_t were determined by Scatchard analyses, as shown in Figure 5.1B.
[b] NT indicates not tested.

Different from membranes of the plant species presented in Table 5.1, membranes isolated from wheat, rice, lettuce, carrot, brussels sprouts and Arabidopsis did not specifically bind ^{125}I-AVR9. Previously, we have shown that the AVR9 binding site is present on the plasma membrane. To confirm that the microsomal membrane fractions contained sufficient plasma membranes, the vanadate-sensitive, K^+ and Mg^{2+}—dependent, H^+-ATPase activities were determined for all fractions used. The H^+-ATPase activities of the membranes of the non-binding plant species were in the same range as the H^+-ATPase activities of the membranes of the plants that do bind AVR9. Thus, the absence of an AVR9 binding site is not due to low quality of

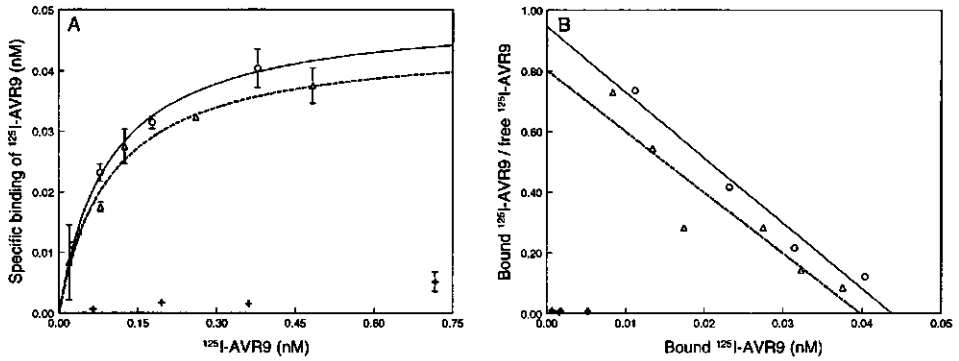

Figure 5.1 Example of saturation curves (A) and Scatchard plots (B) of tomato MM-Cf0 (5 µg microsomal protein, Δ), Oat (5 µg microsomal protein, O) and Arabidopsis, ecotype Landsberg *erecta* (20 µg microsomal protein, +). The K_d and R_t values of all plant species tested were determined by Scatchard analyses and are shown in Table 5.1.

the membranes. Specific H^+-ATPase activities were between 10 and 20 nmol/mg protein/min for microsomal membranes of all plant species tested, except for membranes from barley and wheat, that showed H^+-ATPase activities of ± 500 nmol/mg protein/min.

No correlation between AVR9 binding and presence of Cf-9 *homologues*

DNA gel blot analysis was performed using *Bgl*II-digested DNA of tomato, potato, tobacco, petunia, brussels sprouts, Arabidopsis, wheat, Datura, cucumber, carrot, lettuce, rice, oat and barley. Figure 5.2A shows a blot containing DNA of the various plant species. The blot was hybridized with a probe of the 3' 0.71 kb fragment of *Cf-9*, comprising the last 3 LRRs and the C-terminal part of the CF-9 protein. Following hybridization, and washing in 1 X SSC at 55°C, the lanes containing tomato and potato DNA showed strongly hybridizing bands. The lanes containing tobacco, petunia and Datura DNA showed only weakly hybridizing bands. A smear was observed in the lanes containing wheat, rice, oat, barley and carrot DNA, representing non-specific hybridization. Lanes containing lettuce, brussels sprouts, Arabidopsis and cucumber (not shown) did not show any hybridization. Washing of the blot at less stringent conditions and using prolonged exposure times, revealed a smear in all lanes. At more stringent washing conditions, only the hybridizing bands of the solanaceous plants remained and increasing the washing stringency even

further resulted in hybridizing bands only in the lanes containing tomato and potato DNA. This indicates that DNA fragments with high homology to the *Cf-9* gene are present only in these two plant species. Figure 5.2B shows a DNA blot hybridized with a probe of the 5' 0.65 kb *Bam*H1-*Sac*I fragment of *Cf-9*, comprising the N-terminal part and more than 4 LRRs of the protein. Following hybridization, the blot was washed in 1 X SSC at 55°C. A similar pattern as for the 3' probe was observed, with strong hybridization to DNA of tomato and potato, weak hybridization

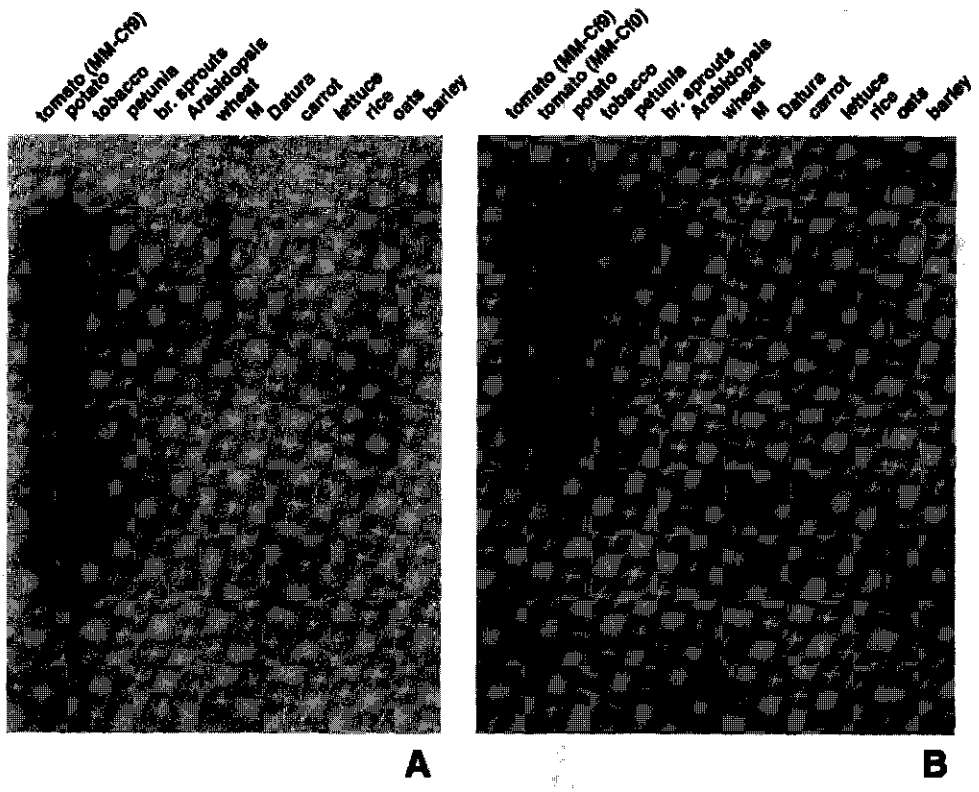

Figure 5.2 **A.** DNA gel blot analysis of different plant species. Genomic DNA was digested with *Bgl*II and 4.5 µg of restricted DNA was used per lane. Following hybridization with the 0.71 kb *Bgl*II-*Eco*RV fragment of the *Cf-9* cDNA as a probe, blot A was washed in 1 x SSC at 55°C. **B.** DNA gel blot hybridized with the 0.65 bp *Bam*H1-*Sac*I fragment of the *Cf-9* cDNA as a probe. Following hybridization blot B was washed in 1 x SSC at 55°C.

to DNA of tobacco, petunia and Datura and hardly any hybridization to DNA of the non-solanaceous plant species. The only non-solanaceous plant species that showed a hybridizing band with the 5' probe was carrot, but this band disappeared at increased washing stringency. A DNA gel blot of cucumber was hybridized with a probe of the 5' *Bam*H1-*Sac*I fragment or with a probe comprising the complete *Cf-9* cDNA. Cucumber DNA showed hardly any hybridization, not even at low stringency washing conditions. This indicates that cucumber does not contain *Cf-9* homologues (results not shown).

No increase in AVR9 binding upon expression of Cf-9 *in tobacco or Arabidopsis*

Tobacco lines, transformed with genomic clones containing the *Cf-9* resistance gene, responded upon injection of AVR9 by showing a gray necrotic lesion at the site of injection (K.E. Hammond-Kosack, and J.D.G Jones, manuscript in preparation for the Plant Cell). Non-transgenic tobacco did not respond upon AVR9 injections. Microsomal membranes of the transgenic tobacco lines containing cosmid pcos34 in either homozygous or heterozygous state and membranes of lines, heterozygous for cosmid pcos110, were tested for their AVR9 binding capacity. Cosmid pcos34 contains the *Cf-9* resistance gene and in addition two upstream *Cf-9* homologues, whereas cosmid pcos110 contains the downstream *Cf-9* homologue in addition to the *Cf-9* resistance gene. The affinity (K_d) of the binding sites for ^{125}I-AVR9 was not significantly different between untransformed tobacco and *Cf-9*—transgenic tobacco lines. As shown in Figure 5.3, the number of AVR9 binding sites (R_t) in *Cf-9*—transgenic tobacco lines was also similar compared to untransformed tobacco. Thus, tobacco, containing *Cf-9* homologues and an active *Cf-9* gene, does not show increased binding of ^{125}I-AVR9.

Transgenic Arabidopsis lines carrying *Cf-9* under control of the CaMV 35S promoter were tested for *Cf-9*—expression using RNA gel blot analysis. This analysis showed that all transgenic plants expressed the *Cf-9* gene. Expression of the *Cf-9* gene was also demonstrated by crossing *Cf-9*—expressing Arabidopsis plants with *Avr9*—expressing Arabidopsis plants, which resulted in seed lethality (Kim Hammond-Kosack, personal communication). However, injections of up to 6 µM of AVR9 into leaves of transgenic Arabidopsis expressing the *Cf-9* gene did not induce necrosis, whereas concentrations down to 0.3 µM induced necrosis in

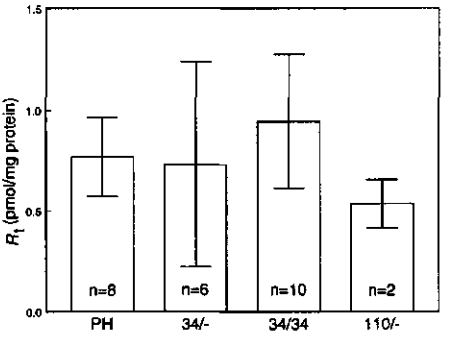

Figure 5.3 Overview of the receptor concentrations (R_ts), of tobacco, cv Petit Havana (PH) and tobacco transformed with cosmid clones containing the *Cf-9* resistance gene. Designation 34/- indicates that the transgenic lines contain cosmid pcos34 in heterozygous state, 34/34 indicates cosmid pcos34 in homozygous state, and 110/- indicates that the lines are transgenic for cosmid pcos110 in heterozygous state. R_t values are in pmol/mg microsomal membrane protein. n indicates the number of binding experiments to membranes from independent transgenic lines or from independent membrane isolations (Petit Havana).

MM-Cf9 tomato leaves (chapter 4). Microsomal membranes of leaves of the 12 transgenic *Cf-9*—expressing Arabidopsis lines were tested for binding of ^{125}I-AVR9. The specific H^+-ATPase activity of these membranes was 8 ± 5 nmol/mg protein/min, indicating that the microsomal membrane fractions contained reasonable quality plasma membranes. No specific binding of AVR9 was observed to membranes of untransformed Arabidopsis plants, nor to membranes of 12 different *Cf-9*—expressing Arabidopsis lines (Figure 5.4). Binding of ^{125}I-AVR9 was not detected, even when the membrane concentration of the transgenic *Cf-9*—expressing Arabidopsis lines was increased from 20 to 50 µg of protein per assay. Thus expression of the *Cf-9* resistance gene in Arabidopsis did not result in binding of AVR9.

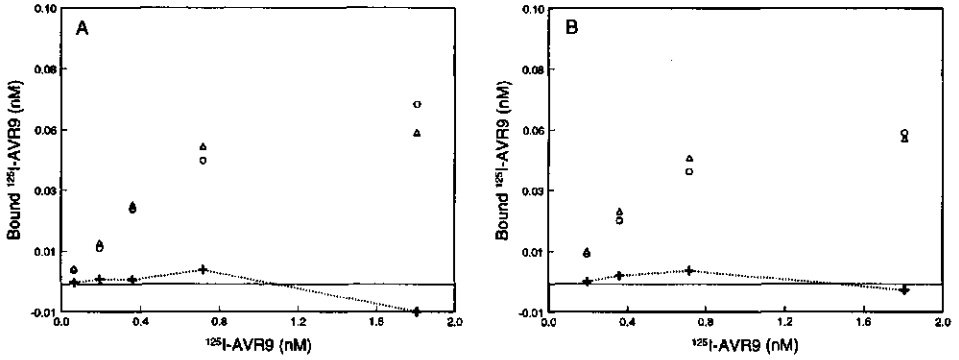

Figure 5.4 Binding of ^{125}I-AVR9 to microsomal membranes of Arabidopsis. **A.** Untransformed Arabidopsis, where Δ represents total binding, O represents background binding, and + represents specific binding. H^+-ATPase—activity of these microsomal membranes was 44 nmol/mg/min. **B.** Arabidopsis transformed with a 35S CaMV—*Cf-9* construct. H^+-ATPase—activity of the microsomal membranes shown was 17 nmol/mg/min.

Discussion

A high-affinity binding site for AVR9 is present in various plant species

A high-affinity binding site for AVR9 is present not only in membranes of tomato, as shown previously (chapter 2), but also in membranes of all other solanceous species tested and in membranes of the non—solanaceous plant species cucumber, barley and oat. The affinity of the binding site (K_d value) of the different plant species is comparable to the K_d value observed for tomato, indicating that the binding sites are identical. Although all calculated K_d values were in the same range, some variation is observed between different membrane isolations. This can be caused by the fact that plants were not grown under standardized conditions and the age of the plants at the time of membrane isolations may have varied. Also, binding of AVR9 to the binding site could be influenced by slight differences in the ionic strength of the membrane preparations (chapter 2). Furthermore, we have used only five different ^{125}I-AVR9 concentrations in our saturation experiments, which could make the Scatchard analyses sensitive to variations.

No correlation between binding of AVR9 and the presence of Cf-9 homologues

We investigated whether homologues of the *Cf-9* resistance gene could encode the high-affinity binding site for AVR9, by comparing the presence of the AVR9 binding site with the presence of *Cf-9*—homologous sequences. Besides membranes of the solanaceous plant species, microsomal membranes from cucumber, barley and oat contain a high-affinity binding site for AVR9. DNA gel blot analyses of these species did not reveal the presence of DNA sequences homologous to the *Cf-9* resistance gene. Thus, we could not show a correlation between the presence of a high-affinity binding site for AVR9 and the presence of homologues of the *Cf-9* resistance gene. Furthermore, only tomato DNA and potato DNA have sequences with high homology to *Cf-9*, whereas membranes of all solanaceous species tested have a high-affinity binding site for AVR9. It is therefore unlikely that the *Cf-9* resistance gene and/or its homologues participate in high-affinity binding of AVR9.

It is possible that only one or a few LRRs of the *Cf-9* resistance gene product or its homologues determine the specificity of AVR9 binding (Windisch et al., 1995). The presence of a sequence encoding only one or a few LRRs in the genome of non—solanaceous plant species would not have been detected in our DNA gel blot analyses. Even LRR proteins of the *Cf-9* structural class, such as *Cf-2*, which shows strong homology to *Cf-9* in the C-terminal 9.5 LRRs (Dixon et al., 1996) and which shows stretches of up to 15 amino acids identical to *Cf-9*, does not cross-hybridize to *Cf-9*. To find further evidence that the *Cf-9* resistance gene does not encode the high-affinity binding site, binding analyses were performed to microsomal membranes from *Cf-9*—transgenic tobacco and Arabidopsis plants, expressing the *Cf-9* gene. Binding of AVR9 to membranes of *Cf-9*—transgenic and non—transgenic tobacco was identical. No alteration in the number of binding sites (R_t-value) or change in binding affinity (K_d-value) was observed in the *Cf-9*—expressing tobacco. Furthermore, AVR9 did not show any binding to membranes of untransformed Arabidopsis, nor to membranes of transgenic Arabidopsis plants, expressing the *Cf-9* gene. These data confirm that the high-affinity binding site for AVR9 is not encoded by the *Cf-9* resistance gene.

The role of CF-9 in AVR9 perception

We have shown that a high-affinity binding site for AVR9 is present in several plant species and is not encoded by the *Cf-9* gene or its homologues. However, it cannot

be excluded that the AVR9 peptide and the product of the *Cf-9* resistance gene can interact with lower binding affinity under different binding conditions. In previous experiments, we have not revealed the presence of an additional binding site for AVR9 in MM-Cf0 or MM-Cf9 membranes, using ^{125}I-AVR9 concentrations up to 10 nM (chapter 2). A binding site with an affinity of more than 100 nM may have remained undetected in our assays. Here, we have used transgenic Arabidopsis plants to show that the *Cf-9* resistance gene does not encode the high-affinity binding site. These Arabidopsis plants, which do not have 'background' binding, will be used in future experiments to detect a possible 'low-affinity'-binding site encoded by the *Cf-9* resistance gene. Therefore, highly-expressing *Cf-9*-transgenic Arabidopsis will be selected and binding assays will be performed to detect a possible 'low-affinity' binding site for AVR9, using different binding conditions and higher concentrations of ^{125}I-AVR9.

Previously, a role for the high-affinity binding site in the induction of the resistance response has been suggested by the correlation between necrosis-inducing activity of mutant AVR9 peptides and their affinity to the described binding site (chapter 4). Furthermore, plants that do not have the high-affinity binding site for AVR9, like *Cf-9*—transgenic Arabidopsis, are unable to give necrosis in leaves, even upon injection of high concentrations of AVR9. Thus, it seems likely that AVR9 is perceived by the high-affinity binding site and upon binding, the CF-9 protein is recruited into the complex. The resulting AVR9-CF-9-binding protein complex will subsequently initiate the signal cascade leading to HR. This hypothesis implies that expression of the *Cf-9* resistance gene in a variety of binding and non—binding plant species should only give necrosis upon AVR9 injections in plants that contain the high-affinity binding site.

Although Arabidopsis does not have a high-affinity binding site for AVR9, surprisingly, crosses between *Cf-9*—expressing Arabidopsis and *Avr9*—expressing Arabidopsis showed *Cf-9*—*Avr9* dependent seed lethality (Kim Hammond-Kosack, personal communication). Seed lethality is not observed upon crossing *Cf-9* and *Avr9*—expressing tomato, where only seedling lethality is observed (Hammond-Kosack et al., 1994; Honée et al., 1995). This suggests either a different expression pattern of the genes involved in elicitor perception in Arabidopsis, or a different recognition mechanism. Isolation of the high-affinity binding site for AVR9 could give insight in the mechanism of AVR9 perception in resistant plants and possibly reveal an interaction between the binding site and the CF-9 protein.

Material and methods

Plant material

All plants were grown under greenhouse conditions, except lettuce and carrot, which were obtained from the local market. Leaves were harvested from tomato (*Lycopersicon esculentum*), cultivar Moneymaker, without *Cf* resistance genes (MM-Cf0), from a near-isogenic line, carrying the *Cf-9* resistance gene (MM-Cf9), from potato (*Solanum tuberosum* cv Bintje), petunia (*Petunia hybrida* cv Blue Magic), tobacco (*Nicotiana tabacum* cv Petit Havana), wheat (*Triticum aestivum*), barley (*Hordeum vulgare* cv Triumph), pepper (*Capsicum annuum* var Lambada), Datura (*Datura stramonium*), cucumber (*Cucumis sativus*), oat (*Avena sativa*), Indica rice (*Oryza sativa*), lettuce (*Lactuca sativa*), carrot (*Daucus carota*), brussels sprouts (*Brassica oleracea*), Arabidopsis (*Arabidopsis thaliana*, ecotypes Columbia and Landsberg *erecta*), and from *Cf-9*—transgenic tobacco and Arabidopsis, which are described below. Microsomal membrane fractions were isolated from ± 50 gram of leaves, essentially as described previously (chapter 2). All isolation steps were performed at 4°C. Briefly, leaves were ground in ice-cold buffer containing 25 mM Tris-HCl, pH 7.5, 250 mM sucrose, 3 mM EDTA, 10 mg/mL fatty acid-free BSA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Microsomal membrane fractions were isolated following filtration through two layers of Miracloth (Calbiochem, La Jolla, CA) and a two-step centrifugation at 10.000 x g, to remove debris and large aggregates, followed by centrifugation of the supernatant at 100.000 x g, to pellet the membrane particles. To determine the concentration of plasma membranes in the microsomal membrane preparations, the plasma membrane—localized, vanadate sensitive, K^+- and Mg^{2+}-dependent H^+-ATPase activity was determined as described previously (chapter 2).

In addition to the membranes of untransformed plants, membranes were isolated from leaves of *Nicotiana tabacum* cv Petit Havana, transformed with binary cosmid clones pcos34 or pcos110, described by K.E Hammond-Kosack, and J.D.G. Jones (manuscript in preparation). Cosmid pcos34 is 18.5 kb in length and carries an intact copy of the *Cf-9* gene, as well as intact copies of two *Cf-9* homologues, located upstream of the *Cf-9* promoter region. Cosmid pcos 110 is 16.5 kb in length and carries an intact copy of the *Cf-9* resistance gene and an intact copy of a *Cf-9* homologue located downstream of the *Cf-9* gene (K.E Hammond-Kosack, and J.D.G. Jones, manuscript in preparation for the Plant Cell). Also, membranes were isolated from *Arabidopsis thaliana* ecotype Columbia, carrying the *Cf-9* resistance gene under transcriptional control of the CaMV 35S promoter.

Iodination of the AVR9 peptide and binding of AVR9 to microsomal membranes

Binding studies were performed essentially as described in chapter 2. Briefly, the 28—amino acid AVR9 peptide was isolated from apoplastic fluid from tomato (MM-Cf5) leaves, infected by an AVR9-producing race of *C. fulvum* (race 5). AVR9 was radiolabeled with iodine-125

by lactoperoxidase treatment and the mono-iodinated peptide was isolated by reversed-phase HPLC, lyophilized, and stored at -20°C. Five microgram of microsomal membrane protein was resuspended in 90 mL of binding buffer (10 mM phosphate buffer, pH 6.0, 0.1% BSA) and pre-incubated at 37°C for 20 min. For the *Cf-9*—transgenic Arabidopsis lines, 20 µg of microsomal membrane protein was used. Binding was initiated by the addition of 10 µL of different concentrations of ^{125}I-AVR9, followed by incubation under gentle shaking in a water bath for 3 hr at 37°C. The final concentrations of ^{125}I-AVR9 used in each experiment were 5.6×10^{-11}, 10^{-10}, 1.8×10^{-10}, 3×10^{-10} and 5.6×10^{-10} M and concentrations were confirmed by scintillation counting. Glass fiber filters (Whatman GF/F) were soaked for 1 to 2 hr. in 0.5% polyethylenimine, and washed with 5 mL of H_2O, and 2 mL of binding buffer. Samples were filtered through these filters at 10^4 Pa followed by washing with 12 mL of binding buffer and radioactivity was counted. All data were collected in duplicate and nonspecific binding was determined in the presence of a 1000—fold excess of unlabeled AVR9. The dissociation constants (K_ds) and receptor concentrations (R_t s), were determined by Scatchard analyses.

DNA gel blot analysis

Genomic DNA for gel blot analysis was isolated from the different plant species as previously described (Van der Beek et al., 1992; wheat, Rogers and Bendich, 1985). DNA was digested with the restriction enzyme *Bgl*II, and the fragments were separated on a 1% agarose gel and blotted onto Hybond-N$^+$ (Amersham, Buckinghamshire, UK) membranes. Different fragments of the *Cf-9* resistance gene were used for hybridization. These included the C-terminal 0.7 kb *Bgl*II-*Eco*RV fragment, comprising the last three LRRs, domains D, E, F, G and part of the poly-A tail of the *Cf-9* gene (domains are described in Jones et al., 1994). Also, a *Bam*H1-*Sac*I fragment (0.65 kb), was used, comprising part of the untranslated region, the signal peptide (A domain), cysteine-rich region (B-domain) and the N-terminal 4 1/3 LRRs. In addition, the complete *Cf-9* cDNA was used as probe on blots containing tomato and cucumber DNA. DNA was labeled with α^{32}P-dATP using the ready-to-go labeling kit from Pharmacia (Woerden, The Netherlands) (Feinberg and Vogelstein, 1983). Hybridization was performed according to the manufacturers' instructions and blots were washed differentially in 3 x SSC (0.15 M NaCl and 0.015 M sodium citrate), 1 X SSC and 0.2 X SSC at 55°C and 0.2 X SSC at 65°C.

Expression of the Cf-9 *resistance gene in transgenic plants*

A functional CF-9 protein was present in all transgenic tobacco plants, as demonstrated by K.E Hammond-Kosack, and J.D.G. Jones (manuscript in preparation). Functional expression of the *Cf-9* gene in leaves of transgenic Arabidopsis lines was tested by pressure infiltration of 6 µM of the AVR9 peptide into these leaves. Furthermore, RNA gel blot analyses were performed. RNA was isolated from Arabidopsis leaves according to Dean et al. (1985). Ten µg of total RNA was used per lane and hybridization was according to Church and Gilbert (1984), using the complete *Cf-9* cDNA as a probe.

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CHAPTER 6

GENERAL DISCUSSION

The research presented in this thesis focused on the question: Does the product encoded by the *Cf-9* resistance gene specifically bind the AVR9 elicitor, thereby initiating a hypersensitive response (HR)? Thus, it was attempted to prove or reject the simplest interpretation of Flor's gene-for-gene hypothesis that predicts that the AVR9 elicitor is recognized by a receptor, encoded by the corresponding *Cf-9* resistance gene (chapter 1; Flor, 1942 and 1971; Gabriel and Rolfe, 1990). This interpretation is presented as Model A in Figure 6.1. A high-affinity binding site for AVR9 was identified and characterized (chapter 2). However, the presence of this binding site is not restricted to tomato plants carrying the *Cf-9* resistance gene, which leads to conclude that the characterized binding protein is not the CF-9 protein, encoded by the *Cf-9* gene (chapter 5). Thus, the initial elicitor-receptor model (Model A) could not be confirmed.

Experiments were designed to determine whether the characterized high-affinity binding site is required to initiate HR. Therefore, amino acids of AVR9 important for necrosis-inducing activity were identified by independently substituting each amino acid by an alanine residue (chapter 3). A correlation between the affinity of the different mutant peptides to the binding site and their necrosis-inducing activity was shown (chapter 4). This correlation suggests that the high-affinity binding site for AVR9 is indeed a functional receptor that initiates the CF-9—dependent HR. Based on these findings, a second model is proposed, presented as Model B in Figure 6.1. In Model B, the AVR9—binding protein 'presents' the AVR9 elicitor to the *Cf-9*—encoded protein or, upon binding of AVR9, recruits CF9 into the complex. Subsequently, CF-9 will initiate the signal cascade resulting in HR by a yet unknown mechanism.

A third model, presented as Model C in Figure 6.1, cannot be excluded based on the generated data. In Model C, AVR9 binds to CF-9 with very low affinity, and CF-9 subsequently initiates HR. However, under the conditions used, a low-affinity binding site has never been detected in our assays. Model C would imply that the high-affinity binding site described in chapter 2 is not required to initiate HR. In the

following sections, research in related fields is discussed and analogies with the proposed models are described.

Elicitor perception in plants

Nowadays, biochemical and genetic information on recognition of race-specific elicitors by plants increases rapidly. For several plant-pathogen interactions it becomes clear that perception of race-specific elicitors by plants is more complex than suggested by the interpretation of the gene-for-gene model presented in Model A. Current knowledge on the perception of pathogen elicitors by plants will be discussed in relation to the models presented in Figure 6.1.

The first cloned gene-for-gene–based resistance gene, *Pto,* encodes a serine-threonine kinase. Pto kinase is the first resistance gene product for which a direct interaction with the matching avirulence gene product has been identified. When the avirulence gene *AvrPto* from *Pseudomonas syringae* pv *tomato* and the *Pto* resistance gene are introduced into the same yeast cell by the two-hybrid system (Fields and Song, 1989), a strong interaction is observed (Tang et al., 1996; Scofield et al., 1996). AvrPto in combination with other Pto-related kinases, including pto kinase (encoded by the recessive *pto*-allele) or the Fen protein (encoded by the dominant *Fen*-allele, conferring sensitivity to the insecticide Fention) showed no interaction. The current model on this interaction states that AvrPto is introduced into the plant cell by the type III secretion system of *P. syringae* (Pirhonen et al., 1996; Bonas and Van den Ackerveken, 1997). Inside the plant cell, AvrPto directly interacts with Pto kinase, leading to activation of a phosphorylation cascade resulting in HR. Although the *in planta* interaction remains to be proven, the interaction between AvrPto and Pto kinase seems to be in agreement with the elicitor-receptor model (analogous to Figure 6.1. Model A, but intracellular). However, in addition to the *Pto* gene, the *Prf* gene, which encodes a leucine-rich repeat (LRR) protein, is essential for induction of HR. Analogous to CF-9 in Model B, the precise role of the Prf protein in AvrPto signaling is unknown, and whether it binds to the Pto-AvrPto complex is unclear.

Another race-specific elicitor of plant defense for which binding studies have been performed is AvrD from *Pseudomonas syringae*. The *avr*D gene encodes an enzyme that releases syringolides, which specifically elicit HR in soybean plants carrying the *Rpg4* resistance gene (Keen, 1996). It is unclear whether syringolides

enter plant cells actively or passively, but HR induction is independent of the type III secretion system. Binding studies using radiolabeled syringolides have shown that a binding site is present in soluble fractions from both resistant *Rpg4* and susceptible *rpg4* soybean cultivars (Ji et al., 1997). The binding affinity of several chemical syringolide derivatives showed good correlation to their elicitor activity in *Rpg4* cell lines, suggesting physiological relevance of the binding site. Native gel binding assays disclosed two bands that bound the labeled syringolide (Ji et al., 1997), whereas affinity columns permitted isolation of a complex of three proteins (Keen, 1996). This suggests that binding of the avrD elicitor is not only independent of the presence of the *Rpg4* resistance gene, but also requires a complex of proteins.

A variant of the strict gene-for-gene–based resistance has been reported for the Arabidopsis resistance gene *RPM1* (Bisgrove et al.,1994). *RPM1* confers resistance to *P. syringae* carrying two very different *Avr* genes, *AvrB* and *AvrRpm1.* Possibly, these Avr proteins bind to different receptors, which both require RPM1 for HR induction. On the other side of the gene-for-gene picture, manipulation of avirulence genes does not only cause loss of avirulence (Joosten et al., 1994; Taraporewala and Culver, 1996), but can also create new avirulence specificities. For instance, the *avrBs3* family of avirulence genes, encodes proteins with a variable number of 34—amino acid repeats (Herbers et al., 1992). Manipulation of the number of these repeats caused loss of original avirulence, but at the same time created new avirulence specificities. This suggests that the resistance genes against these new avirulence gene products are not newly created due to selection pressure by the pathogen, but are present in the population already.

The described variations on the gene-for-gene theme suggest that the simple interpretation of the elicitor-receptor model shown in Model A is insufficient to describe the complex events that lead to recognition of elicitors in plants. Therefore, new models are required to explain the actual recognition event. Model B provides a good candidate to clarify the present knowledge on elicitor recognition.

Ligand perception in mammalian systems.

In mammalian recognition systems, several mechanisms of ligand perception have been described, which show similarities to Model B. For instance, transforming growth factor-β (TGF-ß) requires two distantly related receptors, designated TßR-I

and TβR-II for the initiation of a response. TGF-ß belongs to a family of cystine-knotted peptides that is involved in growth and differentiation. The growth factor family of cystine-knotted peptides is structurally distinct from the inhibitory cystine-knotted peptides, to which AVR9 belongs. Most TGF-ß family members act as disulfide-bonded dimers that signal through complexes of the two receptors TβR-I and TβR-II (Wrana et al., 1994). These receptors have a serine-threonine kinase domain, which has also been found in the resistance genes *Xa21* (Song et al., 1995) and *Pto* (Martin et al., 1993). Besides the intracellular kinase, both TβR-I and TβR-II receptors have a transmembrane region and a small cysteine-rich extracellular domain. TβR-II is a constitutively active kinase, which acts as a 'first' accessory receptor for TGF-β. After ligand binding, TβR-II interacts with the 'second' receptor, TβR-I, which activates the kinase of TβR-I and initiates signal transduction.

The tumor necrosis factor receptor (TNF-R) family comprises of cell surface receptors with no known enzymatic activity. One of the activities of TNF-R family (Itoh et al., 1991) is to initiate programmed cell death, a mechanism that shows similarities to the HR (Dangl et al., 1996). TNF-Rs seem to have a similar signaling mechanism as the TGF-ß family of receptors. It is proposed that TNF-Rs utilize a ligand-passing mechanism to activate signaling, which means that the 'first' accessory receptor recruits the ligand and regulates the rate of association to the 'second' signaling receptor (Tartaglia et al., 1993). Like the extracytoplasmic resistance gene product CF-9 and the TGF-ß receptors, the tyrosine kinase receptors consist of polypeptide chains with a single transmembrane region. Apart from this overall structural appearance, they share little sequence similarities with the predicted resistance gene products. Several of their ligands have a cystine-knot motif (structurally distinct from AVR9). Initiation of signaling of tyrosine kinases occurs by ligand-dependent dimerization or oligomerization, followed by autophosporylation (reviewed by Heldin, 1995). Dimerization does not always involve two identical receptor molecules (homodimers), but may also involve two less related molecules (heterodimers). For epidermal growth factor (EGF), the extracellular domain of these heterodimers can have as little as 36% amino acid identity (Earp et al., 1995). For the fibroblast growth factor (FGF), not only its receptors form dimers or oligomers, but FGF itself can form a complex with an accessory molecule (heparin), upon which the complex binds to receptors, which in turn become oligomerized.

The class I cytokine receptor family coregulates amongst others the immune system. The receptors of this family have a large extracytoplasmic domain and a small intracellular domain with conserved motifs of unknown function. The cloned receptors have low affinity for their ligands and require additional components to form high-affinity binding sites. The receptors form dimers or trimers upon binding of the ligands (Taga and Kishimoto, 1996).

Cell-surface receptor proteins that have intrinsic signaling functions can be utilized by pathogenic agents for their own purpose. An example of this is the Human Immunodeficiency Virus (HIV), the causal agent of AIDS. This virus utilizes (at least) two receptors for entering cells. 'CD4' is known to be the HIV-receptor. Additionally, HIV requires a 7-transmembrane cell-surface chemokine coreceptor (CCR5 or CCR3), to be able to enter the host cell (Moore, 1997).

When comparing the mechanism of recognition of the AVR9 elicitor with recognition systems in mammals, various similarities and common features can be observed. In general, communication or recognition mechanisms include secretion of soluble signaling molecules, that interact with a receptor embedded within the plasma membrane. Signaling cascades can be activated by monomeric, dimeric or oligomeric ligands that activate homodimerization, heterodimerization or complex formation of receptors. Dimerization or complex formation is thought to provide accuracy and flexibility in recognition. Following dimerization, residues on cytoplasmic proteins are phosphorylated by kinases, which can either reside in the receptor itself or in an associated molecule.

Compared to the mammalian communication and recognition mechanisms, relatively limited information is available on recognition of fungal components by plants. The research described in this thesis has led to new models on the recognition of AVR9 and the initiation of HR by tomato plants that carry the *Cf-9* resistance gene. Recognition of AVR9, as shown in Model B, is very similar to recognition of TGF-β and TNF, where the AVR9 high-affinity binding site represents a 'first' accessory receptor, which activates the 'second' receptor CF-9. There is also resemblance between Model B and recognition through tyrosine kinases, where binding to the 'first' receptor can induce heterodimerization with a 'second' receptor protein with very low homology (CF-9). It is unclear whether AVR9 itself can form a dimer or a complex with an accessory molecule before binding, but surface properties of AVR9 reveal no possible sites for dimer formation (Vervoort,

personal communication). There are obvious similarities between recognition of the described mammalian ligands and recognition of the avirulence factor AVR9 by tomato plants. The frequent occurrence of both receptors and accessory receptors in nature, suggests that they represent an important, versatile recognition mechanism.

Conclusion

The research presented in this thesis has given new insight in the recognition of elicitors by plants. We have shown that the 'simple' elicitor-receptor model alone does not accurately describe recognition of the AVR9 elicitor by tomato plants that carry the *Cf-9* resistance gene. It appears that additional components are required for recognition and induction of HR. We have characterized the first component in recognition of the AVR9 elicitor, which is a high-affinity binding site. Several intriguing questions remain to be answered. Where and how does the protein encoded by the *Cf-9* resistance gene initiate the signal transduction pathway? What is the intrinsic function of the high-affinity binding site, besides mediating recognition of AVR9? And what is the intrinsic function of the AVR9 elicitor? Possibly, the AVR9 elicitor peptide has a function in pathogenicity. The various extracellular elicitor peptides produced by *C. fulvum in planta* may provide the fungus with a large array of possibilities to retrieve nutrients from the plant, which might require binding to a receptor. As an unwanted side-effect for the fungus, tomato genotypes that contain the *Cf-9* resistance gene not only bind AVR9, but can also initiate HR. The isolation and characterization of the high-affinity binding site is essential to elucidate the role of AVR9 binding in *Cf-9*—dependent HR induction and could in addition shed light on the intrinsic function of the AVR9 peptide for *C. fulvum*.

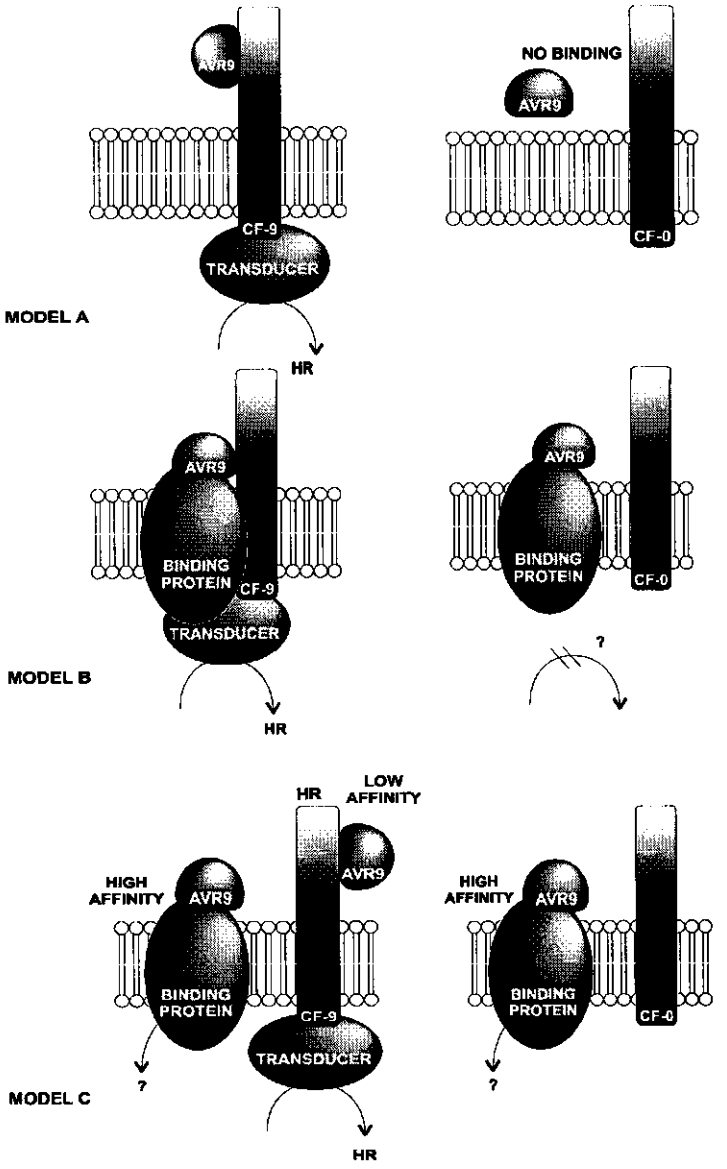

Figure 6.1 Models on perception of the AVR9 elicitor by MM-Cf9 (left) and MM-Cf0 (right) tomato. MM-Cf0 contains homologues of the *Cf-9* resistance gene, of which an encoded protein is designated CF-0. Model **A** represents the elicitor-receptor model. In Model **B** AVR9 binding mediates the interaction between CF-9 and the binding-site—AVR9 complex.. Model **C** is an alternative model, where the high-affinity binding site is not required to initiate HR, but HR is induced by low-affinity binding of AVR9 to CF-9.

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SUMMARY

The interaction between the fungal pathogen *Cladosporium fulvum* and tomato has been used as a model system to study the molecular basis of gene-for-gene relationships. *C. fulvum* is a specialized, biotrophic pathogen, which causes leaf mold on tomato. Under humid conditions conidia of *C. fulvum* germinate and form runner hyphae on the lower side of the leaf. If no resistance genes of the plant match any of the avirulence genes of the fungus, the interaction is compatible and infection will proceed. However, when both a resistance gene and its matching avirulence gene are present, the plant recognizes the fungus and the interaction is incompatible. In an incompatible interaction active defense responses, including the hypersensitive response (HR) are initiated, which inhibit fungal growth effectively. Avirulence genes encode race-specific elicitors, which are present in intercellular washing fluids obtained from compatible interactions of *C. fulvum* and tomato (De Wit and Spikman, 1982). Injection of these intercellular washing fluids in tomato plants resistant to the *C. fulvum* strain from which the washing fluids were obtained, results in specific necrosis at the site of injection. The race-specific elicitor AVR9 was isolated and purified (Scholtens-Toma and de Wit, 1988). AVR9 specifically induces necrosis on tomato genotypes carrying the *Cf-9* resistance gene. The encoding *Avr9* gene was isolated, and it was shown that this gene specifically determines avirulence of *C. fulvum* on tomato plants carrying the *Cf-9* resistance gene (Van den Ackerveken et al., 1992; Marmeisse et al., 1993). The *Avr9* gene encodes a 63—amino acid pre-proprotein containing one potential glycosylation site (Van den Ackerveken et al., 1993). Different forms of the AVR9 elicitor were found, of which the mature AVR9 elicitor of 28 amino acids is predominantly present in *C. fulvum*-infected tomato plants (Van den Ackerveken et al., 1993). The global structure of the AVR9 peptide shows 3 antiparallel β-sheets and 3 disulfide bonds that are arranged in a cystine knot (Vervoort et al., 1997).

In the research project described in this thesis, we studied AVR9 elicitor perception in tomato plants that carry the *Cf-9* resistance gene and compared the results to those obtained with tomato plants lacking this gene. Previously, several research groups had shown that elicitors are recognized through plants receptors, which are localized on the plasma membrane (summarized in *chapter 1*). To find and characterize the receptor for AVR9, the peptide was labeled with iodine-125 and binding to tomato membranes was studied, as presented in *chapter 2*. ^{125}I-AVR9 showed specific, saturable, and reversible

binding to plasma membranes isolated from leaves of the tomato cultivar Moneymaker without *Cf* resistance genes (MM-Cf0) and to membranes from a near-isogenic genotype containing the *Cf-9* resistance gene (MM-Cf9). Binding of AVR9 is characterized by high affinity and low receptor concentration, and thus fulfills several criteria expected for functional receptors (Hulme and Birdsall, 1992). The dissociation constant was determined at 0.07 nM, and the receptor concentration was determined at 0.8 pmol/mg microsomal membrane protein. Binding is highly influenced by pH and ionic strength of the binding buffer and by temperature, indicating the involvement of both electrostatic and hydrophobic interactions. Surprisingly, binding kinetics and binding capacity were identical for membranes of the MM-Cf0 and MM-Cf9 tomato genotype, indicating that the *Cf-9* resistance gene is not required for binding of AVR9. By that time, the *Cf-9* resistance gene was isolated (Jones et al., 1994). *Cf-9* belongs to a gene family and homologues of the *Cf-9* resistance gene are present in both resistant and susceptible tomato genotypes. Two new hypotheses were developed of which the first predicts that not only the *Cf-9* resistance gene, but also homologues of the *Cf-9* gene, encode the high-affinity binding site for AVR9. Only the protein encoded by *Cf-9* itself, designated CF-9, would subsequently initiate the signal transduction cascade resulting in HR. The second hypothesis predicts that the AVR9 binding site is neither CF-9 nor a homologue of CF-9. The binding site proposed in the second hypothesis would bind AVR9 and subsequently recruit the CF-9 protein to initiate HR.

As described in *chapters 3, 4,* and *5*, experiments were performed to prove or reject one of these two hypotheses. To determine whether the high-affinity binding site for AVR9 is indeed a functional receptor, we studied the correlation between binding affinity and necrosis-inducing activity of mutant AVR9 peptides. We determined structure-activity relationships of the AVR9 peptide by independently substituting each amino acid of AVR9 by alanine, using a site-directed mutagenesis approach. In addition, surface-exposed amino acid residues of AVR9 were substituted by other amino acids. Activity of mutant *Avr9* constructs was studied by expressing the constructs in MM-Cf9 tomato plants using the potato virus X (PVX) expression system, and assessing the severity of necrosis induced by each PVX::*Avr9* construct. This allowed direct identification of amino acid residues of AVR9 that are essential for elicitor activity. We identified amino acid substitutions resulting in AVR9 mutants with higher, similar or lower elicitor activity compared to the wild-type AVR9 peptide. Mutants of the amino acid residues Phe21 and Leu24 had completely lost elicitor activity. Necrosis-inducing activity of isolated AVR9 peptides correlated well with the necrosis induced by the corresponding PVX::*Avr9* constructs. It was concluded the PVX expression system is ideally suited to analyze

necrosis-inducing activity of AVR9 peptides. We analyzed whether there is a correlation between elicitor activity of the mutant AVR9 peptides and their affinity to the binding site in membranes of tomato. Therefore, *Nicotiana clevelandii* plants were inoculated with a selection of PVX::*Avr9* constructs and mutant AVR9 peptides were purified from these plants. In addition, some AVR9 peptides were chemically synthesized. Characterization by Electrospray Mass Spectrometry, Circular Dichroism-, and ^{1}H-NMR-spectroscopy revealed that both the *in planta* produced and the synthetic mutant peptides were correctly folded. AVR9 peptides purified from PVX::*Avr9*—infected *N. clevelandii* contained one N-acetylglucosamine, although small amounts of non-glycosylated AVR9 peptides were also detected. The glycosylated AVR9 peptides showed lower affinity to the binding site than the non-glycosylated AVR9 peptides, whereas they did not differ significantly in necrosis-inducing activity. For both the non-glycosylated and glycosylated mutant AVR9 peptides, a positive correlation between their affinity to the membrane-localized binding site and their necrosis-inducing activity in MM-Cf9 tomato was found, i.e. peptides with higher affinity to the binding site showed higher necrosis-inducing activity. This correlation suggested that the characterized high-affinity binding site for AVR9 is indeed a functional receptor that initiates the AVR9-CF-9—dependent HR in MM-Cf9 plants.

In *chapter 5*, we studied whether the *Cf-9* resistance gene or (one of) its homologues code for an AVR9 binding site. We tested binding of AVR9 to microsomal membranes of a variety of solanaceous and non-solanaceous plant species and analyzed these species for the presence of *Cf-9*-homologues. All solanaceous species tested contain homologues of the *Cf-9* resistance gene and membranes of these plants contain a high-affinity binding site for AVR9. However, a high affinity binding site for AVR9 is also present on membranes of the non-solanaceous plant species cucumber, barley and oat, which do not contain homologues of the *Cf-9* resistance gene. Membranes of tobacco, transgenic for the *Cf-9* resistance gene, showed no change in the number of AVR9 binding sites. Arabidopsis does not have a binding site for AVR9 and membranes of Arabidopsis, transgenic for the *Cf-9* resistance gene, also showed no AVR9 binding. From this we concluded that not only the *Cf-9* resistance gene, but also its homologues are not required for high-affinity binding of AVR9. Based on the presented data, we have developed a model, explaining recognition of AVR9 in MM-Cf9 tomato (*chapter 6*). This model predicts that the high-affinity binding protein either 'presents' the AVR9 elicitor to the *Cf-9*-encoded protein or that binding of AVR9 induces a conformational change of the high-affinity binding protein. The latter results in recruitment of CF-9 into the AVR9-receptor complex. Subsequently, signal cascade(s) resulting in HR will be initiated.

SAMENVATTING

De interactie tussen het schimmelpathogeen *Cladosporium fulvum* en tomaat is een modelsysteem voor het bestuderen van de moleculaire basis van de 'gen-om-gen' relatie. *C. fulvum* is een gespecialiseerd, biotroof pathogeen, dat de bladvlekkenziekte van tomaat veroorzaakt. Bij hoge luchtvochtigheid kiemen de sporen van *C. fulvum* en vormen runnerhyfen aan de onderkant van het blad. Als de resistentiegenen van de plant niet overeenkomen met de bijpassende avirulentiegenen van de schimmel, is de interactie compatibel en vindt infectie plaats. Echter, als zowel een resistentiegen als het bijpassende avirulentiegen aanwezig zijn, dan herkent de plant de schimmel en is de interactie incompatibel. Een actief afweermechanisme, de overgevoeligheidsreactie (HR), wordt dan aangeschakeld, en de schimmelgroei wordt geremd. Avirulentiegenen coderen voor fysio-specifieke elicitoren, welke zijn geïdentificeerd in de intercellulaire ruimte van vatbare tomatenplanten geïnfecteerd met *C. fulvum*. De fysio-specifieke elicitor AVR9 is geïsoleerd en gezuiverd (Scholtens-Toma en De Wit, 1988). AVR9 induceert alleen necrose in tomatengenotypen die het *Cf-9* resistentiegen bevatten. Het *Avr*9 gen is geïsoleerd en dit gen veroorzaakt avirulentie van *C. fulvum* op tomatenplanten met het *Cf-9* resistentiegen. Het *Avr*9 gen codeert voor een pre-pro-eiwit van 63 aminozuren, met een potentiële glycosyleringsplaats (Van den Ackerveken et al., 1993). Verschillende vormen van het AVR9 eiwit zijn gevonden, waarvan het rijpe eiwit van 28 aminozuren voornamelijk wordt gevonden in *C. fulvum*-geïnfecteerde tomaat (Van den Ackerveken et al., 1993). Het AVR9 eiwit heeft 3 antiparalelle β-sheets en 3 zwavelbruggen, verbonden in een zogenaamde 'cystine-knot' (Vervoort et al., 1997).

Tijdens het onderzoek beschreven in dit proefschrift is de herkenning van het AVR9 peptide door tomatenplanten met het *Cf-9* resistentiegen onderzocht en vergeleken met tomatenplanten zonder het *Cf-9* gen. Voordien hadden verschillende onderzoeksgroepen aangetoond dat elicitors worden herkend door receptoren, welke zich op de plasmamembraan bevinden (*hoofdstuk 1*). Om de receptor voor AVR9 te vinden, is het eiwit gelabeld met jodium-125 en is binding bestudeerd aan tomatenmembranen, zoals beschreven in *hoofdstuk 2*. ^{125}I-AVR9 bond specifiek, verzadigbaar en reversibel aan plasmamembranen van bladeren van zowel de tomatencultivar Moneymaker (MM-Cf0), als aan membranen van de bijna-isogene lijn MM-Cf9, welke het *Cf-9* resistentiegen bevat. De binding van AVR9 wordt gekenmerkt door hoge affiniteit en lage receptorconcentratie, wat duidt op een functionele receptor (Hulme and Birdsall, 1992). De gevonden dissociatieconstante is 0.07 nM en de receptorconcentratie is 0.8 pmol per milligram membraaneiwit. Binding wordt beïnvloed door pH, ionsterkte van de

bindingsbuffer, en temperatuur, hetgeen wijst op het belang van zowel electro-statische, als hydrofobe interacties bij binding. De kinetiek van de interactie en de hoeveelheid bindingsplaatsen zijn vergelijkbaar voor membranen van MM-Cf0 en MM-Cf9, wat suggereert dat het *Cf-9* resistentiegen niet noodzakelijk is voor binding van AVR9. In 1994 is het *Cf-9* resistentiegen geïsoleerd (Jones et al., 1994). *Cf-9* behoort tot een genfamilie, en homologen van het *Cf-9* resistentiegen zijn aanwezig in zowel vatbare als resistente tomatengenotypen. Twee nieuwe hypothesen zijn ontwikkeld, waarbij de eerste ervan uitgaat dat niet alleen het *Cf-9* resistentiegen, maar ook homologen van *Cf-9* kunnen coderen voor de AVR9 bindingsplaats. Alleen het eiwit dat door *Cf-9* zelf wordt gecodeerd (het CF-9 eiwit), kan vervolgens de signaaltransductie-keten activeren die leidt tot HR. De tweede hypothese voorspelt dat de bindingsplaats voor AVR9 niet wordt gecodeerd door *Cf-9* of *Cf-9* homologen. De bindingsplaats zou AVR9 binden en vervolgens het CF-9 eiwit activeren om de HR te induceren.

Zoals beschreven in de *hoofdstukken 3, 4* en *5*, zijn experimenten ontworpen en uitgevoerd om een van de twee hypothesen te bewijzen of te verwerpen. Om te bepalen of de hoge-affiniteits bindingsplaats voor AVR9 inderdaad een functionele receptor is, hebben we eerst gekeken of er een correlatie is tussen bindingsaffiniteit en necrose-inducerende activiteit van mutante AVR9 eiwitten. Daarvoor is een structuur-functie analyse van het AVR9 eiwit gedaan, waarbij elk aminozuur van AVR9 door middel van plaats-gerichte mutagenese van het *Avr9* gen afzonderlijk vervangen is door alanine. Bovendien werden verschillende aminozuur-residuen aan het oppervlak van het AVR9 eiwit vervangen door andere aminozuren. De activiteit van mutante *Avr9* constructen is bestudeerd middels expressie in MM-Cf9 door middel van het aardappel X virus (PVX) expressiesysteem, gevolgd door het bestuderen van de necrotische symptomen van elk PVX::*Avr9* construct in de tijd. Met behulp van deze methode konden de aminozuur-residuen die meer of minder belangrijk zijn voor necrose-inducerende activiteit direct worden geïdentificeerd. Aminozuursubstituties zijn gevonden die resulteerden in AVR9 mutanten met hogere, vergelijkbare, of lagere necrose-inducerende activiteit dan het ongemuteerde AVR9 wildtype. Mutanten van de aminozuur-residuen Phe21 en Leu24 hadden geen waarneembare necrose-inducerende activiteit. De necrose-inducerende activiteit van geïsoleerde AVR9 peptiden in MM-Cf9 kwam overeen met de necrose-inducerende activiteit van het overeenkomstige PVX::*Avr9* construct in MM-Cf9. Hieruit werd geconcludeerd dat het PVX-expressiesysteem uitstekend geschikt is voor het bepalen van necrose-inducerende activiteit van wildtype en mutante AVR9 peptiden. Vervolgens is bepaald of er een correlatie is tussen de necrose-inducerende activiteit van de mutante eiwitten en de affiniteit van deze eiwitten voor de gekarakteriseerde bindingsplaats. Hiervoor zijn *Nicotiana clevelandii* planten geïnoculeerd met een selectie

van de PVX::*Avr*9 constructen en uit deze planten zijn vervolgens mutante AVR9 peptiden geïsoleerd en gezuiverd. Daarnaast zijn verschillende AVR9 eiwitten gesynthetiseerd. Electrospray massa spectrometrie, 'Circular Dichroism'- en ^{1}H-NMR-spectroscopie wezen uit dat zowel de synthetische als de *in planta* geproduceerde eiwitten correct gevouwen waren. Het merendeel van de AVR9 eiwitten, gezuiverd uit PVX::*Avr*9-geïnfecteerde *N. clevelandii* bevatte één N-actylglucosamine. De geglycosyleerde eiwitten hadden een lagere affiniteit voor the bindingsplaats dan de niet-geglycosyleerde eiwitten, terwijl hun necrose-inducerende activiteit niet significant verschillend was. Voor zowel de niet-geglycosyleerde als de geglycosyleerde mutante AVR9 peptiden werd een positieve correlatie gevonden tussen bindingsaffiniteit en necrose-inducerende activiteit in MM-Cf9. Deze correlatie suggereert dat de gekarakteriseerde hoge-affiniteits bindingsplaats voor AVR9 inderdaad een functionele receptor is in MM-Cf9 planten, die na AVR9 perceptie CF-9 activeert en de CF-9—afhankelijke overgevoeligheidsreactie initieert.

In *hoofdstuk 5* is bekeken of het *Cf-9* resistentiegen of andere genen uit de *Cf-9* genfamilie mogelijk coderen voor de AVR9 bindingsplaats. Hiervoor is binding van AVR9 aan membranen van verschillende planten uit de familie van de *Solanaceae* (nachtschade familie) en aan membranen van plantensoorten uit andere families bestudeerd. De verschillende plantensoorten zijn ook getest op de aanwezigheid van homologen van het *Cf-9* resistentiegen. Alle geteste plantensoorten uit de familie van de *Solanaceae* bevatten homologen van het *Cf-9* gen en membranen van deze plantensoorten bevatten een hoge-affiniteits bindingsplaats voor AVR9. Een hoge-affiniteits bindingsplaat voor AVR9 werd echter ook gevonden in membranen van komkommer, haver en gerst, plantensoorten die geen homologen van het *Cf-9* resistentiegen bevatten. Membranen van transgene tabak, getransformeerd met het *Cf-9* resistentiegen, vertoonden geen verandering in het aantal hoge-affiniteits bindingsplaatsen voor AVR9. Membranen van *Arabidopsis thaliana* (zandraket) hebben geen bindingsplaats voor AVR9, terwijl ook membranen van transgene zandraket lijnen, welke het *Cf-9* resistentiegen tot expressie brengen, geen AVR9 binding vertonen. Uit de beschreven resultaten werd geconcludeerd dat noch het *Cf-9* resistentiegen, noch de homologen van het *Cf-9* gen betrokken zijn bij hoge-affiniteit binding van AVR9. Op basis van de beschreven resultaten is een model ontwikkeld (*hoofdstuk 6*). Dit model voorspelt dat de hoge-affiniteits bindingsplaats voor AVR9, het peptide 'aanbiedt' aan het *Cf-9* genproduct, of dat binding van AVR9 een conformatie-verandering in de hoge-affiniteits bindingsplaats induceert. Vervolgens wordt het *Cf-9* genproduct betrokken in het gevormde complex en wordt de signaal transductieketen geïnitieerd, welke leidt tot de *Cf-9*—afhankelijke overgevoeligheidsreactie.

CURRICULUM VITAE

Miriam Gersmann werd op 10 januari 1965 geboren te Naarden. Na het behalen van het VWO diploma aan het Willem de Zwijger College te Bussum en een korte opleiding op het 'intercollege' te Den Haag, ging ze in 1984 Plantenveredeling studeren aan de Landbouwuniversiteit Wageningen. In 1989 behaalde zij het doctoraal examen met als hoofdvakken Plantenveredeling en Fytopathologie. De praktijktijd bracht zij door bij het CSIRO, Division of Plant Industry, te Canberra, Australië. In 1989 trad zij in dienst van TNO Voeding, bij de afdeling Moleculaire Planten Biotechnologie, Centrum voor Fytotechnologie, RUL-TNO te Leiden, waar zij werkte aan de transformatie van gerst, binnen het project 'Adaptation of Barley for Industrial Needs' (ABIN1). In 1993 werd zij aangesteld bij de vakgroep Fytopathologie van de Landbouwuniversiteit op een door NWO gefinancierd project, waarvan de resultaten in dit proefschrift staan beschreven. Sinds augustus 1997 is zij werkzaam als 'postdoc' bij het Centrum voor Plantenveredelings- en Reproduktieonderzoek, waar zij werkt aan een onderzoek naar resistentiegenen tegen *Peronospora parasitica* in Arabidopsis.