The Cf-4 and Cf-9 resistance proteins of tomato:

molecular aspects of specificity and elicitor perception

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Stellingen

1. Dat resistentie-eiwitten waarschijnlijk 'op wacht' staan bij het virulentiedoelwit van een virulentiefactor is een logisch gevolg van natuurlijke selectie.
   Dit proefschrift.

2. Hoewel de grote onderlinge homologie van resistentiegenen uit verschillende plantenfamilies anders doet vermoeden, zijn resistentiegenen meestal niet functioneel tussen verschillende plantenfamilies overdraagbaar.
   Dit proefschrift; Tai et al. (1999) Proc. Natl. Acad. Sci. USA 96, 14153-14159.

3. Omdat het 'birth-and-death' model vrijwel niets met het 'trench-warfare' model te maken heeft, is het onjuist deze begrippen als elkaars synoniemen te beschouwen.
   Kamoun (2001) Curr. Opin. Plant Biol. 4, 295-300.

4. De termen 'virulentiegen' en 'avirulentiegen' duiden op verschillende functies, maar betreffen verrassend genoeg vaak één en hetzelfde gen. Deze terminologie is verwarrend en zou herzien moeten worden.

5. Het zeer intensief onderzochte resistentiegen *Pto* is feitelijk geen resistentiegen.
   Dit proefschrift; Van der Biezen & Jones (1998) Trends Biochem. Sci. 23, 454-456.

6. 'Boom-and-bust' cycli in de moderne landbouw tonen aan dat een goed begrip van de relatie tussen plant en pathogeen in natuurlijke populaties onmisbaar is bij de ontwikkeling van duurzame landbouw.
   Dit proefschrift; Zhu et al. (2000) Nature 406, 718-722.

7. Het bestaan van resistentiegenen in planten, mensen en insecten suggereert dat de strijd tegen ziekteverwekkers reeds lang op dezelfde wijze geleverd wordt.
   Aderem & Ulevitch (2000) Nature 406, 785-787; Inohara et al. (2001) J. Biol. Chem. 276, 2551-2554.

8. In de wetenschap is fantasie onmisbaar, maar deze voorspelt helaas zelden de werkelijkheid.

9. Misverstanden over e-mails ontstaan door afwezigheid van intonatie en visueel contact.

Stellingen behorende bij het proefschrift van
Renier van der Hoorn getiteld:
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Contents

Chapter 1 7
General introduction and outline

Chapter 2 23
Agroinfiltration is a versatile tool that facilitates comparative analyses of *Avr9/Cf-9*-induced and *Avr4/Cf-4*-induced necrosis. Mol. Plant-Microbe Interact. 13, 439-446 (2000).

Chapter 3 39
Identification of distinct specificity determinants in resistance protein Cf-4 allows construction of a Cf-9 mutant that confers recognition of avirulence protein AVR4. Plant Cell 13, 273-285 (2001).

Chapter 4 61
Intragenic recombination generated two distinct *Cf* genes that mediate AVR9 recognition in the natural population of *Lycopersicon pimpinellifolium*. Proc. Natl. Acad. Sci. USA 98, 10493-10498 (2001).

Chapter 5 77
The C-terminal dilysine motif for targeting to the endoplasmic reticulum is not required for Cf-9 function. Mol. Plant-Microbe Interact.14, 412-415 (2001).

Chapter 6 85
Efficient solubilization of the high-affinity binding site for AVR9 from tomato membranes. To be submitted for publication.

Chapter 7 103
General discussion. Modified version submitted for publication.

Summary 121

Samenvatting 125

Dankwoord 129

List of publications 133

Curriculum Vitae 135

Chapter 1

General Introduction and Outline

Renier A. L. van der Hoorn

General Introduction

Ever since the first crop was grown in the prehistoric Middle East, mankind reluctantly learned that plant pathogens take their share. Sometimes a plant pathogen even destroyed the entire crop, causing food shortage, starvation and decimation of the human population. The battle between the farmer and plant pathogens is already going on for several millennia and many successful measures to protect crops have been taken. However, even today, a quarter of all food production is lost due to pests and diseases. With a world population exceeding 7 billion people, food supply has now become one of the biggest challenges facing mankind. In the agriculture of today, chemicals are often successfully used to suppress pests and diseases. However, most chemicals are no longer desired since they can seriously threaten natural ecosystems. Therefore, the search for novel, more sustainable ways to protect crops against pathogens is of great importance. Especially the exploitation of natural defence mechanisms offers great opportunities in producing environmentally safe food of high quality.

Natural resistance of plants against pathogens

In addition to the presence of passive barriers, plants mount active defence responses upon recognition of an attacking pathogen. The most common mechanism associated with active plant defence is the hypersensitive response (HR), during which cells surrounding the infection site quickly die. Active defence mechanisms also include the oxidative burst, deposition of callose, accumulation of pathogenesis–related proteins, accumulation of phytoalexins and many other responses (Hammond-Kosack and Jones, 1996).

The induction of active defence responses is preceded by recognition of the invading pathogen. In many cases, recognition is based on the presence of matching dominant genes in the plant and pathogen. This was first described for the pathosystem of flax and flax-rust (Flor, 1942). A plant with a resistance (*R*) gene will only recognise an invading pathogen if this pathogen carries the matching avirulence (*Avr*) gene. Later, it has been suggested that products of these two genes directly or indirectly interact, resulting in the induction of defence responses (Keen, 1990). Understanding the mechanism of this gene-for-gene recognition event is crucial for the full exploitation of resistance genes to protect crop plants against pathogens.

The tomato-*Cladosporium fulvum* pathosystem

The interaction between tomato plants and the fungus *Cladosporium fulvum* is a well-known model system to study gene-for-gene recognition events (Joosten and De Wit, 1999). *Cladosporium fulvum* is a biotrophic fungus that causes leaf mould on tomato (Figure 1A). During its whole lifecycle, the fungus does not penetrate plant cells, but grows in the extracellular space of tomato leaves (Figure 1B). As a result, all communication between the fungus and its host plant occur in the extracellular space. Tomato leaf mould has been a serious threat for tomato growers and therefore several resistance genes, designated *Cf* genes, have been introgressed from wild tomato relatives into cultivated tomato, such as the cultivar MoneyMaker (MM) (Boukema et al., 1980). Cultivars like MM-Cf4 and MM-Cf9 are fully resistant to strains of *C. fulvum* that carry the *Avr4* or *Avr9* genes, respectively (Figure

1C). Resistance is accompanied by the induction of an HR in the immediate vicinity of the site where the fungus enters the plant (Figure 1D).

As with many new, gene-for-gene-based resistance genes that were introgressed into crop plants, *Cf* resistance genes were eventually overcome by 'new' strains of the fungus. For example, a strain designated 'race 4' appeared that was able to colonise MM-Cf4 tomato plants. The availability of fungal strains of different races and near-isogenic MM tomato lines with different *Cf* genes, has been instrumental to start basic research on the molecular aspects of gene-for-gene-based avirulence and resistance in this particular pathosystem.

AVR4 and AVR9 avirulence proteins of *Cladosporium fulvum*

A major breakthrough in research on gene-for-gene recognition event was obtained with the identification of products of *Avr* genes of the *C. fulvum*, the so-called race-specific elicitors. During growth of the fungus on a susceptible tomato plant, several fungal and plant proteins are secreted into the extracellular spaces of tomato leaves. Washing fluids collected from the extracellular spaces made it possible to identify these components (De Wit et al., 1986). Injection of these apoplastic fluids into leaves of resistant plants appeared sufficient to trigger HR (De Wit and Spikman, 1982). This bio-assay served as a basis for the purification of the elicitors AVR9 and AVR4, which are recognised by MM-Cf9 or MM-Cf4 tomato plants, respectively (De Wit et al., 1985, Scholtens-Toma and De Wit, 1988; Joosten et al., 1994). The elicitor proteins were (partially) sequenced and their corresponding genes isolated by reverse genetics (Van Kan et al., 1991; Joosten et al., 1994). Transformation of virulent fungal races with *Avr4* or *Avr9* rendered them avirulent on MM-Cf4 or MM-Cf9 tomato, respectively (Joosten et al., 1994; Van den Ackerveken et al., 1992). Thus, the *Avr4* and *Avr9* genes are both required and sufficient to confer avirulence of *C. fulvum* on MM-Cf4 or MM-Cf9 tomato plants, respectively. Races that circumvent *Cf-4*- and *Cf-9*-mediated resistance appeared to lack a functional avirulence gene. The *avr4* alleles present in strains virulent on MM-Cf4 plants contain mutations that truncate or destabilise the AVR4 elicitor protein (Joosten et al., 1994 and 1997), whereas the *Avr9* gene is absent in strains that are virulent on MM-Cf9 plants (Van Kan et al., 1991).

Both *Avr4* and *Avr9* encode pre-pro-proteins that contain an N-terminal signal peptide for extracellular targeting (Figure 2). Once secreted into the extracellular space, the pro-proteins are further processed by fungal and/or plant proteases into mature proteins of 87 and 28 amino acids, respectively (Joosten et al., 1997; Van den Ackerveken et al., 1993). AVR4 and AVR9 contain eight and six cysteines, respectively, which are involved in disulfide bridges that stabilise the elicitor proteins (Vervoort et al., 1997, Joosten et al., 1997). The amino acid sequence of the two proteins shows no significant homology with known proteins and their role in virulence of the fungus remains unclear.

Cf-4 and Cf-9 resistance proteins

The *Cf-9* resistance gene was cloned by transposon-tagging in the offspring of a cross between MM-Cf9 tomato and *Avr9*-transgenic tomato (Jones et al., 1994). Seedlings of this cross normally die upon expression of both *Avr9* and *Cf-9* (Hammond-Kosack et al., 1994), but survive if the *Cf-9* gene is inactivated by a transposon. The *Cf-4* gene has been mapped on a locus that is allelic to *Cf-9* (Balint-Kurti et al., 1995) and was subsequently isolated using map-based cloning (Thomas et al., 1997). Transformation of MM-Cf0 tomato

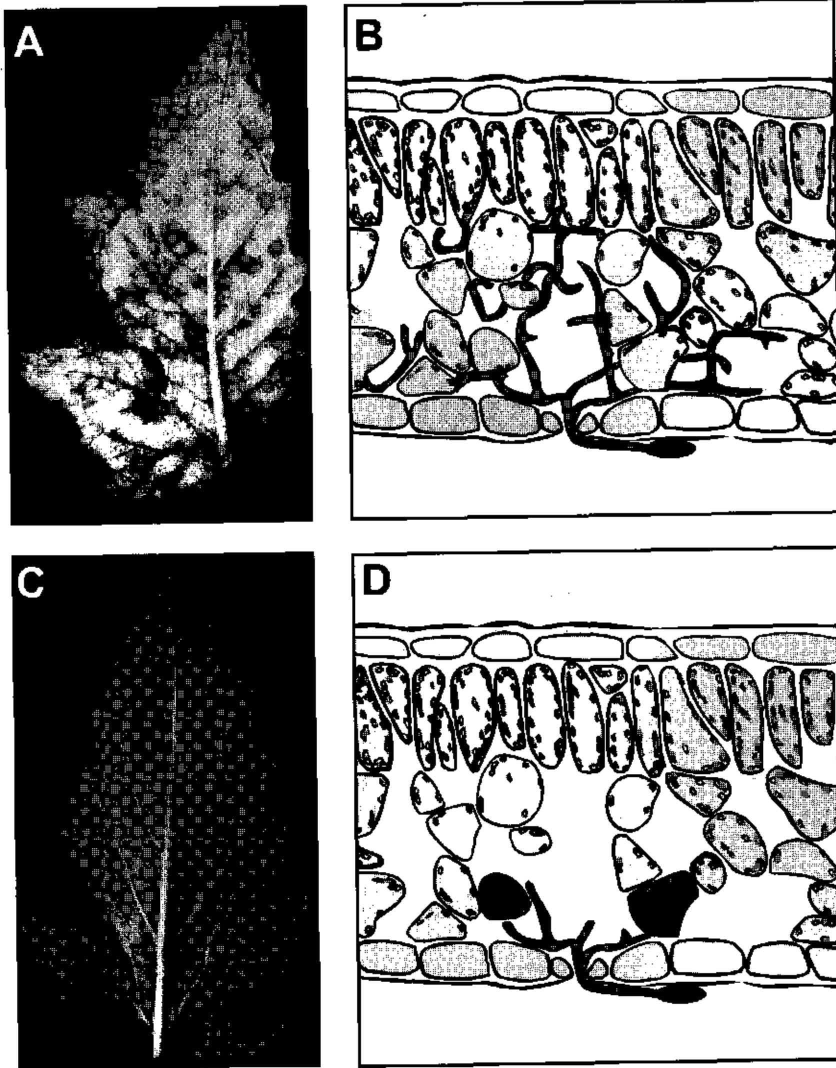

Figure 1. The compatible and incompatible interaction between tomato and *Cladosporium fulvum*.

A Lower side of a leaf of a susceptible tomato plant, two weeks after inoculation with a virulent strain of *C. fulvum* (compatible interaction).

B Schematic representation of a cross section of a susceptible leaf after inoculation with a virulent strain of *C. fulvum*. The 'runner' hyphe has entered the leaf mesophyll through open stomata. During colonisation of the leaf, the mycelium remains confined to the extracellular space.

C Lower side of a leaf of a resistant tomato plant, two weeks after inoculation with an avirulent strain of *C. fulvum*.

D Schematic representation of a cross section of a leaf of a resistant plant after inoculation with an avirulent strain of *C. fulvum*. The fungus is recognised as soon as a hyphe enters a stoma. Recognition results in a hypersensitive response (indicated as dark cells) that restricts further fungal growth.

AVR4	AVR9
MHYTTLLLSTLLVGTALAQP	MKLSLLSVELALLIATTLPL
TNPPAKTPK**KAPKTQPYNPC**	CWAAALPVGLGVGLD**YCNSS**
KPQEVIDTKCMGPKDCLYPN	**CTRAFDCLGQCGRCDFHKLQ**
PDSCTTYIQCVPLDEVGNAK	**CVH**
PVVKPCPKGLQWNDNVGKKW	
CDYPNLSTCPVKTPQPKPKK	
GGVGGKKASVGHPGY	

Figure 2. Amino acid sequence of the elicitor proteins (AVRs) encoded by the *Avr4* and *Avr9* avirulence genes of *Cladosporium fulvum*.
Underlined, signal peptide for extracellular targeting; bold, mature protein; boxed, cysteine residues.

plants with *Cf-4* or *Cf-9* resulted in plants that acquired the ability to recognise the AVR4 and AVR9 proteins, respectively (Hammond-Kosack et al., 1998; Thomas et al., 1997). Also tobacco and potato plants transformed with the *Cf-9* gene became capable of recognising AVR9, visualised by the development of specific necrosis upon injection of AVR9 (Hammond-Kosack et al., 1998).

The amino acid sequences of the proteins encoded by the *Cf-4* and *Cf-9* genes are highly similar (Figure 3) (Jones et al., 1994; Thomas et al., 1997). Both proteins contain a putative signal peptide for extracellular targeting (A-domain), which is followed by a cysteine-rich B-domain, a leucine-rich repeat (LRR) domain (C-domain), a D-domain without conspicuous features, an acidic E-domain, a putative transmembrane domain (F-domain) and a short, basic G-domain. The structure of the domains predicts that the Cf proteins are anchored in the plasma membrane with domains B-E being extracytoplasmic and the G-domain cytoplasmic. Consistent with this topology, the proteins contain a number of putative glycosylation sites in the extracytoplasmic domain (Figure 3). Although a location at the plasma membrane is expected for proteins that are involved in recognition of extracellular AVR proteins, the G-domain contains a C-terminal dilysine motif (KKxx) that can function as a signal for retrieval or retention of membrane proteins to the endoplasmic reticulum (ER) (Teasdale and Jackson, 1996).

The largest part of the Cf-4 and Cf-9 proteins consists of LRRs. Insight in the structure of LRR domains has come from the crystal structure of ribonuclease inhibitors (Kobe and Deisenhofer, 1993), which consist of 15 LRRs and specifically bind, and thereby inhibit, ribonucleases (Kobe and Deisenhofer, 1994). Each LRR contains an xxLxLxx consensus that is predicted to fold as a ß-sheet, with the conserved leucines protruding in the hydrophobic core of the protein, whereas the side chains of the adjacent amino acids (x) are solvent-exposed (Figure 4). In multiple LRRs, the ß-sheets are aligned in parallel and form a surface that is decorated with solvent-exposed residues. It is this side of the protein that is expected to interact specifically with other proteins (i.e. ligands or interactors). The fact that Cf-4 and Cf-9 differ predominantly at solvent-exposed positions (Figure 3) is consistent with this theory. With their predicted structure, a role for Cf proteins as receptors for fungal ligands can be expected. However, the predicted cytoplasmic domain of Cf proteins lacks known signalling motifs that could transduce a signal to the cytoplasm upon AVR perception.

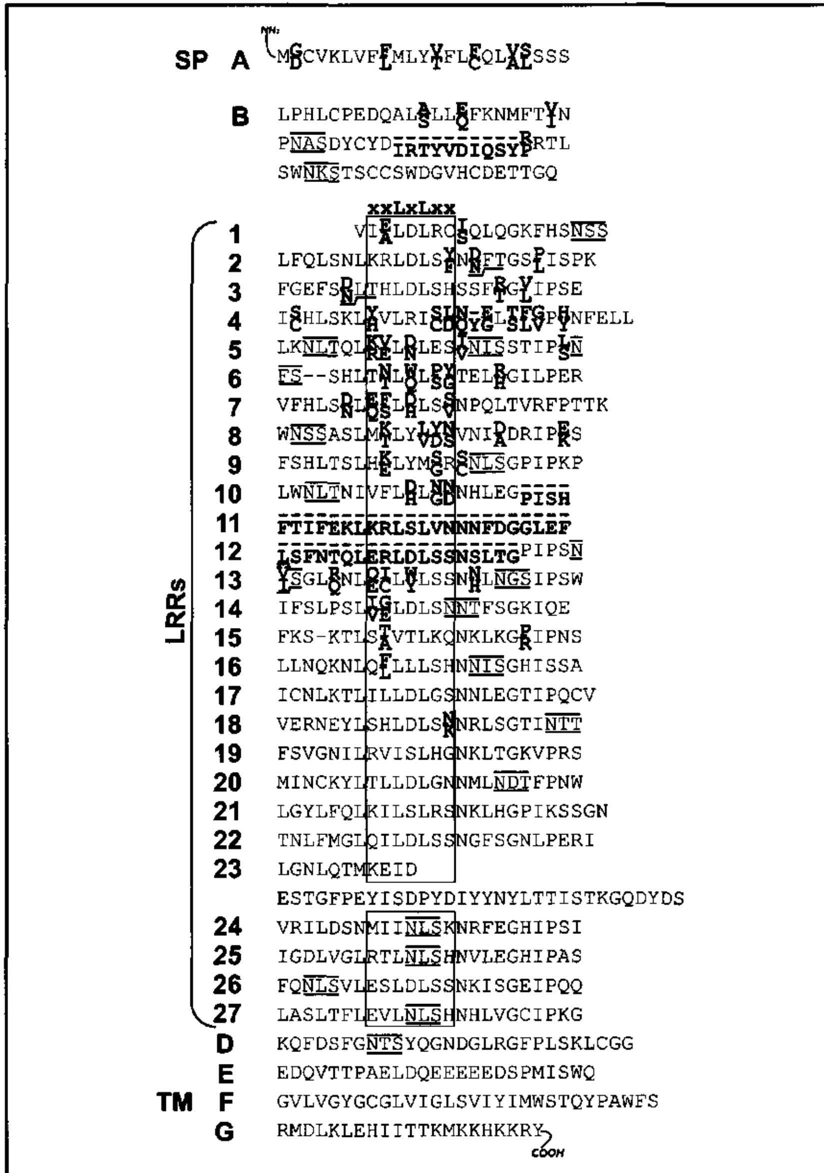

Figure 3. Amino acid sequences of the proteins encoded by the *Cf-4* and *Cf-9* resistance genes of tomato. Amino acid residues of Cf-4 and Cf-9 that are identical are shown in normal script. Cf-4- and Cf-9-specific residues are shown in bold at top and bottom line, respectively. Potential N-glycosylation sites (NxS/T) *in Cf-4 and Cf-9 are overlined and underlined, respectively.* The box indicates the various ß-sheets (consensus xxLxLxx), each of which contains five putative solvent-exposed amino acid residues (x). Domains (indicated on the left) are as follows: SP, signal peptide (A-domain); B, cysteine-rich domain; 1 to 27, LRRs (C-domain); D, domain without conspicuous features; E, acidic domain; TM, putative transmembrane domain (F-domain); G, basic domain representing the putative cytoplasmic tail with putative ER-retrieval signature (KKxx).

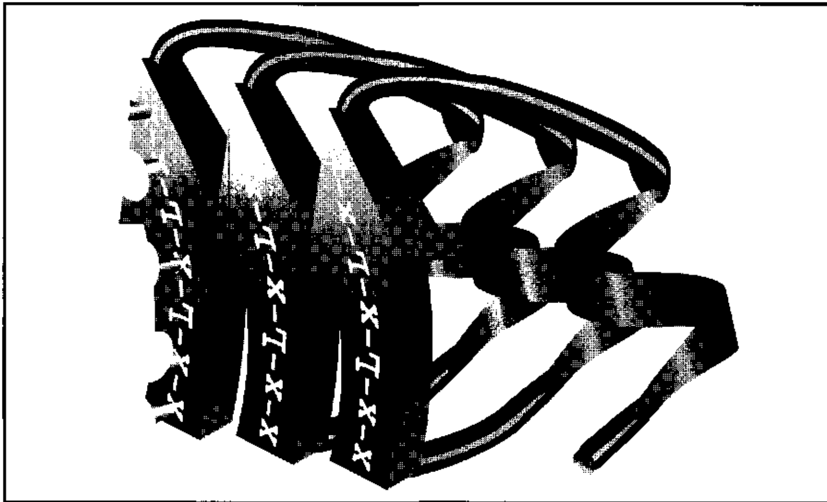

Figure 4. Proposed structure of a section of the LRR domain of Cf proteins.
Each LRR module contains a motif that folds as an α-helix, and the xxLxLxx motif that folds as a ß-sheet. The leucine residues (L) form a hydrophobic core, whereas the side chains of the flanking amino acid residues (x) are solvent-exposed. In multiple LRRs (three are shown), the ß-sheets are aligned in parallel and form a surface decorated with solvent-exposed residues that can interact specifically with a ligand.

With respect to their structure and predicted localisation, the Cf proteins are distinct from other classes of R gene products that have been described so far (Table 1 and Figure 5). Most classes of R genes encode proteins with a nucleotide-binding site (NBS) and LRRs, and are predicted to reside in the cytoplasm, where they are likely involved in direct of indirect interaction with ligands produced by the attacking pathogen that are present in the host cytoplasm (De Wit, 1997; Van der Biezen and Jones, 1998). The tomato Pto resistance gene is exceptional since it encodes a serine/threonine kinase that lacks LRRs (Martin et al., 1993). However, Pto function depends on the presence of Prf, which encodes an NBS-LRR protein (Salmeron et al., 1996). The Xa21 resistance gene from rice encodes a transmembrane receptor with extracellular LRRs and a cytoplasmic kinase (Song et al., 1995). R proteins like Hs1^{pro-1} and Pi-ta have a leucine-rich domain in which a typical LRR signature cannot be identified (Bryan et al., 2000; Cai et al., 1997).

The *Cf-4* and *Cf-9* resistance genes of tomato are present at loci that consist of clusters of homologous genes (*Hcr9*s: Homologues of *Cladosporium fulvum* resistance gene *Cf-9*). The *Cf-4* and *Cf-9* loci are allelic and each contains five *Hcr9* genes, of which *Cf-4* represents *Hcr9-4D* and *Cf-9* represents *Hcr9-9C* (Figure 6) (Parniske et al., 1997). The *Hcr9-4E* gene, which is located on the *Cf-4* locus directly downstream of the *Cf-4* gene, is also a functional *Cf* resistance gene that confers recognition of fungal strains carrying the *Avr4E* gene (Takken et al., 1998 and 1999). At the *Cf-9* locus at least one additional *R* gene to *C. fulvum*, presumably *Hcr9-9B*, is present (Parniske et al., 1997; Laugé et al., 1998).

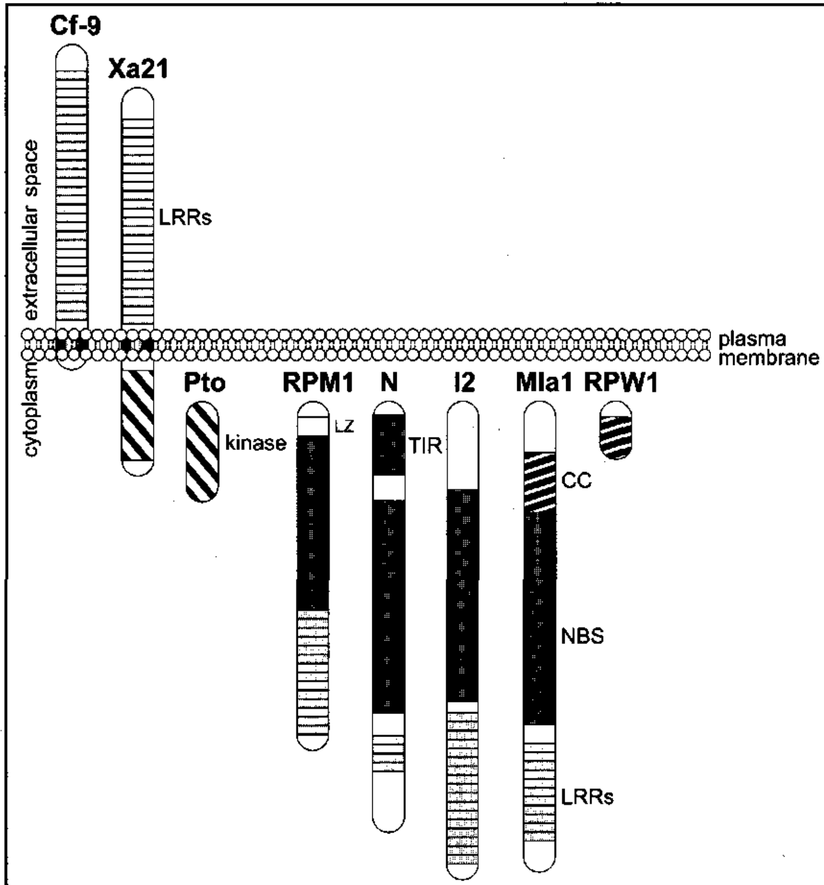

Figure 5. Schematic representation of different classes of resistance proteins.
One R protein of each class is represented. LRRs, leucine-rich repeats; TIR, Toll/Interleukin-like receptor domain; LZ, leucine zipper; NBS, nucleotide-binding site; CC, coiled-coil structure. For further details see Table I.

The *Cf-4/9* clusters both map on the short arm of chromosome 1, at a locus called *'Milky Way'*. Additional *Hcr9* genes map at the *'Northern Lights'* (5 *Hcr9s*) and *'Southern Cross'* (2 *Hcr9s*) loci (Parniske et al., 1998) (Figure 6). Sequence analysis of the *Hcr9* genes revealed that they consist of sequence 'patch works', suggesting that they have evolved by sequence exchange between homologous genes (Parniske et al., 1997 and 1999).

AVR9 binding studies

Biochemical interpretation of the gene-for-gene model predicts that AVR proteins directly interact with R proteins. Consistent with this hypothesis, as described above, *Avr4* and *Avr9* of *C. fulvum* encode ligand-like extracellular proteins and the matching *Cf* resistance genes encode receptor-like proteins, which are presumably located in the plasma membrane of tomato cells. Along this line, experiments were designed to prove whether a

Table1. Overview of cloned resistance genes.

R[1]	plant[2]	pathogen[3]
Pto	tomato	*P. syringae* pv. *tomato*
Prf	tomato	*P. syringae* pv. *tomato*
RPS2	Arabidopsis	*P. syringae* pv. *tomato*
RPM1	Arabidopsis	*P. syringae* pv. *maculicola*
RPS5	Arabidopsis	*P. syringae* pv. *phaseolicola*
RPP8	Arabidopsis	*Pernospora parasitica*
Mi	tomato	*Meloidogyne incognita*
		Macrosiphum euphorbia
Rx	potato	Potato Virus-X
Rx2	potato	Potato Virus-X
Gpa2	potato	*Globodera pallida*
HRT	Arabidopsis	Turnip Crincle Virus
RPP13	Arabidopsis	*Peronospora parasitica*
I2c	tomato	*Fusarium oxysporum*
I2	tomato	*Fusarium oxysporum*
Xa1	rice	*X. oryzae* pv. *oryzae*
Sw-5	tomato	*tospovirus*
Rp-1D	maize	*Puccinia sorghi*
Dm3	lettuce	*Bremia lactucae*
Pib	rice	*Magnaporthe grisea*
Bs2	pepper	*X. campestris* pv. *vesicatoria*
Cre3	wheat	*Heterodera avenae*
Pi-ta	rice	*Magnaporthe grisea*
N	tobacco	Tobacco Mosaic Virus
RPP1	Arabidopsis	*Peronospora parasitica*
L6	flax	*Melampsora lini*
M	flax	*Melampsora lini*
RPP5	Arabidopsis	*Peronospora parasitica*
RPS4	Arabidopsis	*P. syringae* pv. *pisi*
P	flax	*Melampsora lini*
RPP10	Arabidopsis	*Peronospora parasitica*
RPP14	Arabidopsis	*Peronospora parasitica*
Xa21	rice	*X. oryzae* pv. *oryzae*
Cf-9	tomato	*Cladosporium fulvum*
Cf-2	tomato	*Cladosporium fulvum*
Cf-4	tomato	*Cladosporium fulvum*
Cf-5	tomato	*Cladosporium fulvum*
Hcr9-4E	tomato	*Cladosporium fulvum*
9DC	tomato	*Cladosporium fulvum*
Hs1^{pro-1}	sugar beet	*Heterodera schachtii*
Mla1	barley	*Blumeria graminis*
Mla6	barley	*Blumeria graminis*
RPW8	Arabidopsis	*Erysiphe cruciferarum*

[1] Resistance gene; [2] plant from which *R* gene has been isolated; [3] pathogen towards which the *R* gene is targeted; [4] domains within R proteins. LRR, leucine-rich repeat domain; LR, leucine-rich domain; TIR, Toll/Interleukin-like receptor domain; LZ, leucine zipper; CC, coil-coiled domain; NBS, nucleotide-binding site; PK, protein kinase; TM, transmembrane domain. [5] reference; [6] matching avirulence gene (if cloned). Table has been updated until July 2001.

type	reference[5]	cloned *Avr*[6]
PK	Martin et al., 1993, Science 262, 1432	*avrPto*
LZ-NBS-LRR	Salmeron et al., Cell 86, 123 (1994)	*avrPto*
LZ-NBS-LRR	Bent et al., Science 265, 1856 (1994)	*avrRpt2*
	Mindrinos et al., Cell 78, 1089 (1994)	
LZ-NBS-LRR	Grant et al., Science 269, 843 (1995)	*avrB/avrRpm1*
LZ-NBS-LRR	Warren et al., Plant Cell 10, 1439 (1998)	*avrRps5*
LZ-NBS-LRR	McDowell et al., Plant Cell 10, 1861 (1998)	
LZ-NBS-LRR	Milligan et al., Plant Cell 10, 1307 (1998)	
	Rossi et al., PNAS 95, 9750 (1998)	
LZ-NBS-LRR	Bendahmane et al., Plant Cell 11, 781 (1999)	*PVX-CP*
LZ-NBS-LRR	Bendahmane et al., Plant J. 21, 73 (2000)	
LZ-NBS-LRR	Van der Vossen et al., Plant J. 23, 567 (2000)	
LZ-NBS-LRR	Cooley et al., Plant Cell 12, 663 (2000)	*TCV-CP*
LZ-NBS-LRR	Bittner-Eddy et al., Plant J. 21, 177 (2000)	
NBS-LRR	Ori et al., Plant Cell 9, 521 (1997)	
NBS-LRR	Simons et al., Plant Cell 10, 1055 (1998)	
NBS-LRR	Yoshimura et al., PNAS 95, 1663 (1998)	*avrXa1*
NBS-LRR	Brommonschenkel et al., MPMI 10, 1130 (2000)	
NBS-LRR	Collins et al., 1999, Plant Cell 11, 1365 (1999)	
NBS-LRR	Meyers et al., Plant Cell 10, 1817 (1998)	
NBS-LRR	Wang et al., Plant J. 19, 55 (1999)	
NBS-LRR	Tai et al., PNAS 96, 1453 (1999)	*avrBs2*
NBS-LRR	Lagudah et al., Genome 40, 659 (1997)	
NBS-LR	Bryan et al., Plant Cell 12, 2033 (2000)	*AvrPita*
TIR-NBS-LRR	Whitham et al., Cell 78, 1101 (1994)	*TMV-helicase*
TIR-NBS-LRR	Botella et al., Plant Cell 10, 1847 (1998)	
TIR-NBS-LRR	Lawrence et al., Plant Cell 7, 1195 (1995)	
TIR-NBS-LRR	Anderson et al., Plant Cell 9, 641 (1997)	
TIR-NBS-LRR	Parker et al., Plant Cell 9, 879 (1997)	
TIR-NBS-LRR	Gassmann et al., Plant J. 20, 265 (1998)	*avrRps4*
TIR-NBS-LRR	Dodds et al., Plant Cell 13, 163 (2001)	
TIR-NBS-LRR	Botella et al., Plant Cell 10, 1847 (1998)	
TIR-NBS-LRR	Botella et al., Plant Cell 10, 1847 (1998)	
LRR-TM-PK	Song et al., Science 270, 1804 (1995)	
LRR-TM	Jones et al., Science 266, 789 (1994)	*Avr9*
LRR-TM	Dixon et al., Cell 84, 451 (1996)	*Avr2*
LRR-TM	Thomas et al., Plant Cell 9, 2209 (1997)	*Avr4*
LRR-TM	Dixon et al. Plant Cell 10, 1915 (1998)	
LRR-TM	Takken et al., Plant J. 14, 401 (1998)	*Avr4E*
LRR-TM	Van der Hoorn et al., this thesis, chapter 4	*Avr9*
LR-TM	Cai et al., Science 275, 832 (1997)	
CC-NBS-LRR	Zhou et al., Plant Cell 13, 337 (2001)	
CC-NBS-LRR	Halterman et al., Plant J., 25, 335 (2001)	
CC	Xiao et al., Science 291, 118 (2001)	

direct interaction indeed occurred between the AVR and Cf proteins. Binding studies with radiolabeled AVR9 showed the presence of a specific, high-affinity binding site (HABS) for AVR9 in plasma membranes isolated from tomato (Kooman-Gersmann et al., 1996). However, this HABS was also present in tomato plants that lacked the *Cf-9* gene. Thus, the *Cf-9* gene itself does not encode the HABS. The HABS is not only present in microsomal fractions of all tomato genotypes tested, but was also detected in all solanaceous plants that were tested and in a number of other plant species (Kooman-Gersmann et al., 1996). However, no HABS was detected in plants such as Arabidopsis, lettuce and various Brassica species (Kooman-Gersmann, 1998).

In addition, many experiments have been performed to obtain evidence that AVR9 directly interacts with Cf-9, maybe with low affinity. For this purpose, *Cf-9* was expressed in plants or insect and mammalian cell cultures, but in none of these cases, interaction with AVR9 could be demonstrated (Luderer et al., 2001). Although it has not been possible to prove that there is no interaction, it seems unlikely that AVR9 directly interacts with Cf-9, even with low affinity. This suggests that other components, like the HABS, are required in the AVR9-Cf-9 receptor complex.

For the involvement of the HABS in AVR9 perception in tomato containing the *Cf-9* gene evidence, although indirect, has been obtained. It was found that different necrosis-inducing activities of AVR9 mutant peptides, directly correlate with their affinity for the HABS (Kooman-Gersmann et al., 1998). In addition, *Cf-9*-transgenic Arabidopsis, which lacks the HABS, fails to respond to AVR9 (Kooman-Gersmann, 1998). Vice versa, for tobacco, potato and tomato, that contain the HABS (Kooman-Gersmann et al., 1996), introduction of *Cf-9* is sufficient to render these plants responsive towards AVR9 (Hammond-Kosack et al., 1998). These observations point to a crucial role for the HABS in AVR9 recognition by MM-Cf9 tomato plants and suggest that the HABS and Cf-9 are present in one active receptor complex (Joosten and De Wit, 1999).

Outline of the thesis

To examine the role of the various domains of Cf proteins in perception of AVR proteins of *C. fulvum* in more detail, a functional, transient expression system was developed for the *Cf-4* and *Cf-9* resistance genes. **Chapter 2** describes such an expression system for *Cf* genes, based on infiltration of tobacco leaves with Agrobacterium strains that carry *Cf* genes on the T-DNA of binary plasmids (agroinfiltration). Established assays were included to examine the reliability and versatility of the agroinfiltration assay. In addition, agroinfiltation allowed comparison between *Avr9/Cf-9*- and *Avr4/Cf-4*-induced necrosis. Finally, agroinfiltration was used to examine the function of *Avr/Cf* gene pairs in heterologous plant species.

The agroinfiltration assay is an excellent expression system to study the effect of mutations in *Cf* genes. In **chapter 3**, agroinfiltration was used to determine specificity determinants in Cf proteins by exchanging domains between Cf-4 and Cf-9 and subsequently examining the effect of these mutations on specificity of perception of AVR proteins. The importance of specific features in the B-domain and the number of LRRs was examined, as well as the role of Cf-4- and Cf-9-specific amino acids. Specificity determinants in Cf-4 were identified, allowing the construction of a Cf-9 mutant carrying the specificity determinants of Cf-4.

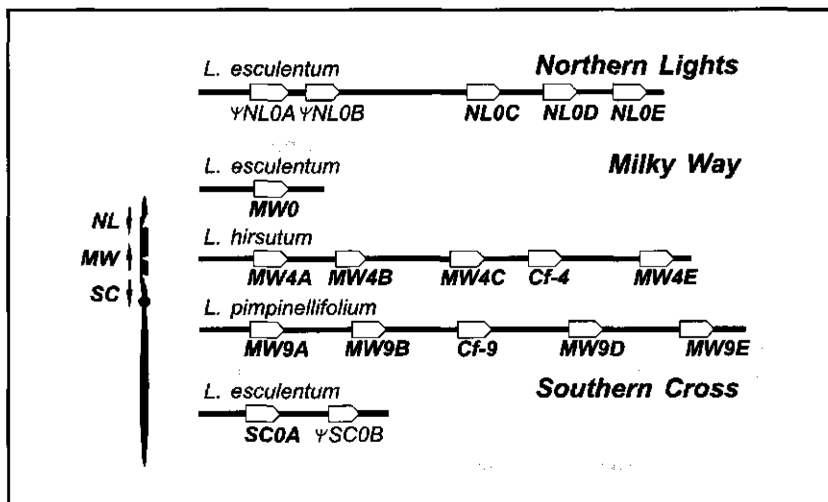

Figure 6. Map position and physical structure of *Hcr9* gene clusters.
On the left, a schematic genetic map of the short arm of tomato chromosome 1 is shown, depicting the position of three *Hcr9* loci (*Northern Lights (NL)*, *Milky Way (MW)* and *Southern Cross (SC)*) relative to each other. Open arrows indicate the position and transcriptional polarity of the *Hcr9* (pseudo) genes. Figure is adapted from Parniske et al. (1999).

To learn more about the flexibility of specificity determinants of Cf-9 proteins, we examined the molecular variation of Cf-9 in natural populations of *Lycopersicon pimpinellifolium (Lp)*, from which the *Cf-9* locus has been introgressed into cultivated tomato (**chapter 4**). Also the spread and frequency of AVR9 recognition in this population was determined. Genes conferring AVR9 recognition from plants of different geographical regions were characterised in detail, and their frequency and polymorphism were determined.

Having examined their specificity determinants, we subsequently focused on other motifs in Cf proteins. The presence of a dilysine motif in the G-domain of Cf-9 (KKRY) suggests that the protein resides in the endoplasmic reticulum. In **chapter 5** we examined the effect of mutations in the KKRY motif on the function of Cf-9. The presented data explain the data presented by two previous publications.

As it is likely that both the high-affinity binding site (HABS) for AVR9 and the Cf-9 protein reside in the plasma membrane and may be present in the same receptor complex, it is essential to isolate the HABS in order to get more insight in the molecular mechanism of specific AVR9 perception. In **chapter 6**, a procedure is described that allows solubilisation of the HABS without affecting its AVR9-binding activity. This procedure may become an important tool to study the AVR9 receptor complex at the biochemical level.

In the final chapter (**chapter 7**), the experimental data presented in the previous chapters are discussed in a broader context. The role of additional host factors in Avr protein perception in other gene-for-gene interactions is reviewed and explained from an evolutionary point of view. This chapter also predicts future directions in research on plant-pathogen interactions and provides new directions for crop protection in modern agriculture.

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Chapter 2

Agroinfiltration Is a Versatile Tool That Facilitates Comparative Analyses of *Avr9/Cf-9*-Induced and *Avr4/Cf-4*-Induced Necrosis

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Agroinfiltration Is a Versatile Tool That Facilitates Comparative Analyses of *Avr9/Cf-9*-Induced and *Avr4/Cf-4*-Induced Necrosis

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Abstract

The avirulence genes *Avr9* and *Avr4* from the fungal tomato pathogen *Cladosporium fulvum* encode extracellular proteins that elicit a hypersensitive response when injected into leaves of tomato plants carrying the matching resistance genes, *Cf-9* and *Cf-4*, respectively. We successfully expressed both *Avr9* and *Avr4* genes in tobacco with the Agrobacterium transient transformation assay (agroinfiltration). In addition, we expressed the matching resistance genes, *Cf-9* and *Cf-4*, through agroinfiltration. By combining transient *Cf* gene expression with either transgenic plants expressing one of the gene partners, Potato Virus X (PVX)-mediated *Avr* gene expression, or elicitor injections, we demonstrated that agroinfiltration is a reliable and versatile tool to study *Avr/Cf*-mediated recognition. Significantly, agroinfiltration can be used to quantify and compare *Avr/Cf*-induced responses. Comparison of different *Avr/Cf*-interactions within one tobacco leaf showed that *Avr9/Cf-9*-induced necrosis developed slower than necrosis induced by *Avr4/Cf-4*. Quantitative analysis demonstrated that this temporal difference was due to a difference in *Avr* gene activities. Transient expression of matching *Avr/Cf* gene pairs in a number of plant families indicated that the signal transduction pathway required for *Avr/Cf*-induced responses is conserved within solanaceous species. Most non-solanaceous species did not develop specific *Avr/Cf*-induced responses. However, co-expression of the *Avr4/Cf-4* gene pair in lettuce resulted in necrosis, providing the first proof that a resistance (*R*) gene can function in a different plant family.

Introduction

Co-evolution between plants and pathogens has enabled plants to develop effective surveillance systems to recognise pathogens and mount defence responses. Defence responses are diverse and usually include a hypersensitive response (HR) where tissue surrounding the infection site becomes necrotic (Hammond-Kosack and Jones, 1996). The plant surveillance system has a genetic basis, involving dominant resistance (*R*) genes that confer the ability to recognise invading pathogens carrying matching avirulence (*Avr*) genes. Tremendous efforts in the past decade have resulted in the cloning of many *R* and *Avr* genes.

R gene products can be broadly classified into two groups based on their predicted cellular location (De Wit, 1997; Jones and Jones, 1997; Parker and Coleman, 1997).

The first and largest group of R proteins is cytoplasmic and the members often contain leucine-rich repeats (LRRs) and nucleotide binding sites (NBSs). Members of this group have been cloned from flax (e.g., *L* genes), lettuce (*Dm* genes), Arabidopsis (e.g., *RPP* genes), several solanaceous species (e.g., *N, Mi, Gpa, Bs2*), and monocots (*Xa1* and *Cre3*) (reviewed by Van der Biezen and Jones, 1998). The *Pto* gene, which encodes a serine-threonine kinase, is the only cytoplasmic R protein within this group that lacks LRRs and NBSs. The second and smaller group of *R* genes encodes putative plasma membrane-anchored proteins. They all carry extracellular LRR domains and members of this group have been cloned from rice (*Xa21*), sugar beet (*Hs1^{pro-1}*), and tomato (e.g., *Cf-9* and *Cf-4*) (Cai et al., 1997; Jones and Jones, 1997).

Proteins that are encoded by *Avr* genes share less common features (Culver et al., 1991; Laugé and De Wit, 1998; Van den Ackerveken and Bonas, 1997). Their predicted cellular location often fits with that of their matching *R* gene product. For example, the *Avr9* and *Avr4* genes from the biotrophic leaf mold fungus *Cladosporium fulvum* encode elicitor proteins that are secreted into the tomato leaf apoplast. Injection of these elicitor proteins into extracellular leaf spaces of tomato plants that carry a matching *Cf* gene is sufficient to trigger an HR (Joosten and De Wit, 1999). In contrast, viral and bacterial AVR proteins only elicit an HR when produced in the host cytoplasm and not when injected into leaves (Bonas and Van den Ackerveken, 1997). The latter proteins possibly interact with *R* gene products in the host cytoplasm, as was shown for the AvrPto and Pto proteins (Scofield et al., 1996; Tang et al., 1996).

To improve our understanding of *Avr/Cf* interactions at the molecular level, transient expression with Potato virus X (PVX; Chapman et al., 1992) has been employed to study the effects of mutations in *Avr9* and *Avr4* genes (Joosten et al., 1997; Kooman-Gersmann et al., 1997). However, transient expression of *Cf* genes through PVX is constrained by the size of the inserted gene that is allowed in the recombinant virus. In contrast to PVX, Agrobacterium can accommodate large genes and has a broad host range (Bundock and Hooykaas, 1998). Transient expression of genes through infiltration of Agrobacterium cultures into leaf tissue (agroinfiltration) is a quick and easy method to study genes of interest (Kapila et al., 1997; Rossi et al., 1993).

In this report, we demonstrate that *Avr9* and *Avr4*, as well as their large matching *R* genes *Cf-9* and *Cf-4*, respectively, can be successfully expressed by agroinfiltration. We show that agroinfiltration can be combined with either transgenic plants expressing one of the matching gene partners, PVX-mediated *Avr* gene expression, or injection of elicitor protein. In addition, we used agroinfiltration for quantitative analysis and comparison of different *Avr/Cf*-induced responses in tobacco and other plant species. Although initial results indicated that *Avr9/Cf-9*-induced necrosis developed slower than that induced by *Avr4/Cf-4*, we demonstrate that this temporal difference is due to differences in *Avr* gene activities. Transient expression of matching gene partners in a number of plant species revealed that the signal transduction pathway required for *Avr/Cf*-induced responses is conserved within solanaceous species. Most non-solanaceous species did not show specific *Avr/Cf*-induced responses, with the exception of lettuce, in which necrosis was induced by co-expression of the *Avr4/Cf-4* gene pair.

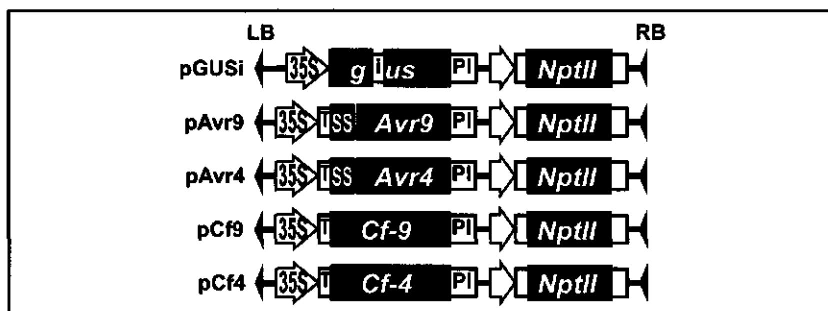

Figure 1. Schematic representation of the T-DNAs present on the five binary plasmids used in this study.
Each binary plasmid is named after the gene that is present on the T-DNA. The β-glucuronidase (*gus*) gene is interrupted by an intron, which excludes Agrobacterium-derived *gus* expression (Vancanneyt et al., 1990). *Avr9* and *Avr4* are fused to the tobacco pathogenesis-related *PR1a* signal sequence to ensure extracellular targeting. Symbols: black boxes, ORFs; open boxes, untranslated regions; open arrows, promoters; black triangles, T-DNA borders. Abbreviations: *Gus*, gene encoding β-glucuronidase; i, intron; *Avr9*, ORF encoding 28 amino acid mature AVR9 protein; *Avr4*, ORF encoding 86 amino acid mature AVR4 protein; SS, signal sequence from tobacco pathogenesis-related *PR1a* gene; *Cf-9*, ORF encoding wild-type Cf-9; *Cf-4*, ORF encoding wild-type Cf-4; 35S, Cauliflower Mosaic Virus (CaMV) 35S promoter; T, omega Tobacco Mosaic Virus (TMV) leader; PI, potato proteinase inhibitor-II polyadenylation region; RB, right border of T-DNA; LB, left border of T-DNA; *NptII*, neomycin-phosphotransferase II.

Results

Transient expression of *Avr* and *Cf* genes in tobacco

As tobacco can be transformed easily and *Cf-9* transgenic tobacco was found to respond with an HR upon injection with AVR9 protein (Hammond-Kosack et al., 1998), we used *Cf-9* transgenic tobacco to transiently express the *Avr9* gene through agroinfiltration. When young, fully expanded leaves were infiltrated with Agrobacterium carrying pAvr9 (Figure 1), the entire infiltrated area became necrotic (Figure 2A). Leaf tissue started to collapse at 1-day post infiltration (dpi) and had developed into a yellow-brown sector by 7 dpi. In wild-type tobacco no necrosis occurred upon transient *Avr9* expression (Figure 2B). Similarly, transient expression of *Avr4* resulted in necrotic sectors in *Cf-4* transgenic tobacco but not in wild-type tobacco (data not shown). A major advantage of agroinfiltration is that the T-DNA can accommodate large genes such as the 2.6-kb open reading frame (ORF) of the *Cf-9* resistance gene. Transient expression of *Cf-9* in *Avr9* transgenic tobacco (Hammond-Kosack et al., 1994) resulted in necrosis (Figure 2C), while no necrotic responses were induced in wild-type tobacco (Figure 2B). Together, these results demonstrate that genes that encode the extracellular elicitors AVR9 or AVR4 and the large, extracellular, membrane-anchored Cf-9 protein can be successfully expressed in tobacco through agroinfiltration.

Both *Avr9* and *Avr4* have been transiently expressed through the PVX expression system (Hammond-Kosack et al., 1995; Joosten et al., 1997). To test whether transient *Cf* gene expression through agroinfiltration can be combined with PVX-mediated *Avr* gene expression, wild-type tobacco plants were inoculated with PVX::*Avr9*, PVX::*Avr4*, or wild-

type PVX. Two weeks after PVX inoculation, Agrobacterium carrying pCf9 or pCf4 (Figure 1) was infiltrated into leaves that showed clear mosaic symptoms. Necrosis only appeared in sectors where matching gene pairs were expressed (Figure 2D). This indicates that agroinfiltration of both *Cf-9* and *Cf-4* genes can successfully be combined with PVX-mediated expression of the matching *Avr* gene.

Elicitor peptides AVR9 and AVR4 were originally purified from apoplastic fluids (AFs) isolated from compatible *C. fulvum*-tomato interactions (Scholtens-Toma and De Wit, 1988; Joosten et al,. 1994). In addition to these proteins, AFs contain many other fungal elicitor proteins. To test the specificity of tobacco leaves that transiently express *Cf-9* or *Cf-4* for AVR9 and AVR4 detection, respectively, crude AFs were injected 1 day after agroinfiltration of wild-type tobacco with pCf9 or pCf4. Necrotic responses were only detected in leaf sectors that were injected with AFs containing a matching elicitor (Figure 2E), indicating that tobacco tissue that transiently expresses *Cf* genes has the same specificity for recognition of AVR proteins as tomato genotypes containing the native *Cf* genes.

Moneymaker-Cf9 (MM-Cf9) tomato leaves are very sensitive to injection of AVR9 elicitor protein, as concentrations of AVR9 as low as 300 nM result in a clear necrotic response (Kooman-Gersmann et al., 1998). When tobacco leaves that transiently express the *Cf-9* gene were injected with a concentration series of AVR9 peptide, concentrations as low as 10 nM resulted in a clear necrotic response above background (Figure 2F). As expected, only background responses developed in tissue that transiently expressed *Cf-4* (Figure 2F). This suggests that transient *Cf-9* expression levels in tobacco are high enough to detect low concentrations of AVR9.

Comparison of necrotic responses induced by transient expression of *Avr9/Cf-9* and *Avr4/Cf-4* gene pairs

To date, comparisons between *Avr9/Cf-9*- and *Avr4/Cf-4*-induced responses have not been conclusive, due to developmental and/or genetic differences between plants that harbour the different *Cf* genes. The ability to express *Cf* genes simultaneously in the same leaf tissue enabled us for the first time to compare induced responses within the same leaf. Therefore, Agrobacterium cultures carrying the pAvr and pCf plasmids were mixed in a 1:1 ratio and infiltrated into wild-type tobacco leaves. Necrosis only developed in leaf sectors that expressed matching gene pairs (Figure 3A). Clear differences between *Avr9/Cf-9*- and *Avr4/Cf-4*-induced responses were observed. Tissue collapse induced by expression of the *Avr9/Cf-9* gene pair occurred 1 day later than that induced by the *Avr4/Cf-4* gene pair (Figure 3B). Typically, *Avr9/Cf-9*-induced necrosis only started to develop after the entire *Avr4/Cf-4*-expressing area had collapsed. Although the pattern of tissue collapse was identical, the *Avr9/Cf-9*-induced collapse was preceded by weak chlorosis. The colour of the necrotic sector resulting from *Avr9/Cf-9* co-expression gradually turned dark brown (Figure 3A), suggesting that there was time for the accumulation of phenolic compounds.

Although the binary constructs used in this study were comparable, differences observed in the speed of necrotic responses could be caused by differences in activities of the pAvr or pCf plasmids upon agroinfiltration. We therefore quantified responses induced upon infiltration of a dilution series of Agrobacterium carrying pAvr and pCf (Figures 3C and 3D). To exclude differences between culture densities, cultures of equal density that carry matching pAvr and pCf plasmids were mixed in different ratios. The percentage of

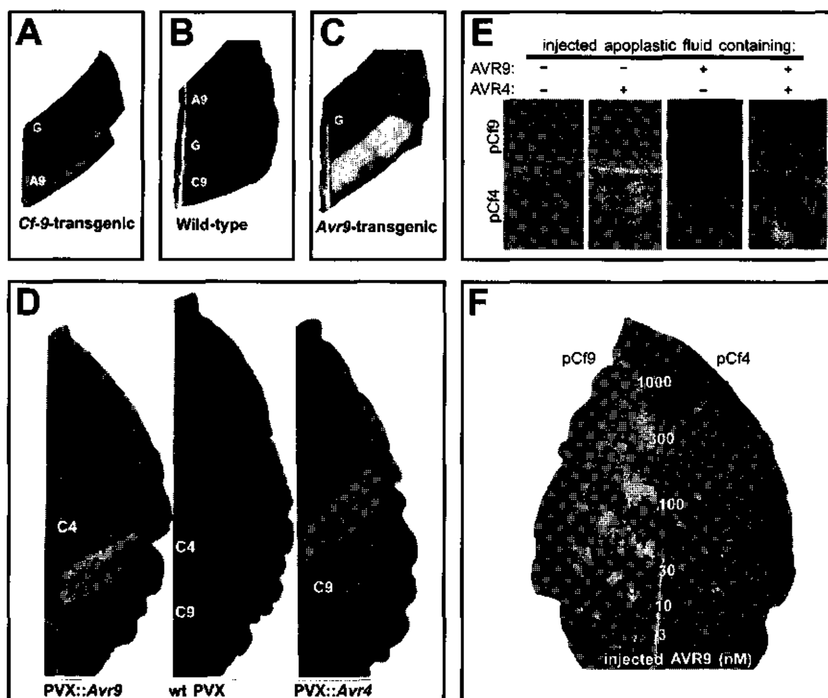

Figure 2. Transient expression of *Avr* and *Cf* genes in tobacco leaves. Agrobacterium carrying pGUSi (G), pAvr9 (A9), pCf9 (C9), or pCf4 (C4) was infiltrated into young, fully expanded leaves of 6-week-old tobacco plants. Photographs were taken at 7 days post infiltration (dpi).
A Transient expression of the β-glucuronidase (*gus*) gene and *Avr9* in *Cf-9* transgenic tobacco.
B Transient expression of *Avr9, gus,* and *Cf-9* in wild-type tobacco.
C Transient expression of *gus* and *Cf-9* in *Avr9* transgenic tobacco.
D Transient *Cf* gene expression in Potato Virus X (PVX)-inoculated, wild-type tobacco plants. Two weeks post inoculation, cultures of Agrobacterium carrying pCf9 or pCf4 were infiltrated into young, fully expanded leaves that showed mosaic symptoms caused by PVX.
E Injection of apoplastic fluids (AFs), isolated from different compatible *C. fulvum*-tomato interactions into tobacco leaves at 1 dpi with Agrobacterium containing pCf9 or pCf4. AFs were isolated from tomato cultivar Moneymaker Cf0 inoculated with *C. fulvum* race 2.4.5.9.11 (MM-Cf0/race 2.4.5.9.11) (lacks AVR9 and AVR4, first panel), MM-Cf5/race 2.5.9 (lacks AVR9, second panel), MM-Cf4/race 2.4.8.11 (lacks AVR4, third panel), and MM-Cf0/race 5 (contains both AVR9 and AVR4, fourth panel).
F Injection of a concentration series of AVR9 protein, performed at 1 dpi with Agrobacterium containing pCf9 (left leaf half) or pCf4 (right leaf half) into wild-type tobacco leaves. Photograph was taken at 7 dpi.

infiltrated leaf area that had become necrotic at 7 dpi was measured and plotted against the percentage of Agrobacterium cultures that carry pAvr and pCf. The percentage of the culture containing pCf that induced 50% necrosis (NC^{50}) of the infiltrated leaf area was calculated from two independent experiments. NC^{50} values for pCf9 were 1.86 and 3.74%, respectively, whereas NC^{50} values for pCf4 were calculated as 1.38 and 4.92%, respectively (Figure 3C). This indicates that pCf9 and pCf4 have comparable activities. NC^{50} values for

pAvr9 and pAvr4 were calculated as 2.56 ± 0.88% and 0.27 ± 0.12%, respectively (n = 4, Figure 3D), indicating that pAvr9 has a 10-fold lower activity, compared with pAvr4. Significantly, at concentrations corresponding to these NC^{50} values no difference in timing between Avr9/Cf-9- and Avr4/Cf-4-induced necrosis was observed (data not shown). These data indicate that the temporal differences in necrotic responses induced by Avr9/Cf-9 and Avr4/Cf-4 gene pairs when cultures were mixed in a 1:1 ratio are caused by differences in activities between pAvr plasmids upon agroinfiltration.

Transient expression of matching *Avr/Cf* gene pairs in different plant families

The extensive homology between *R* gene products suggests that signal transduction cascades that lead to disease resistance are highly conserved between plant families. To examine whether species other than tobacco and tomato have the signal transduction components that are required for *Avr/Cf*-induced responses, we transiently co-expressed matching gene pairs in a number of different plant species. Transient expression of the β-glucuronidase (*gus*) gene served as an indication of the transformation efficiency and the level of gene expression. Specific responses that were induced by co-expressing matching gene pairs were compared with aspecific responses induced by *gus* expression and by co-expressing non-matching gene pairs.

Some plant species were difficult to infiltrate (e.g., soybean, rice, and maize) whereas others showed very low levels of GUS staining (e.g., sugar beet, broad bean, and Brussels sprouts) (data not shown). Plant species that showed severe background responses included tomato, potato, cucumber, and pepper (data not shown). Therefore, agroinfiltration in these plant species remains to be optimised.

Plant species that showed significant GUS staining and low background responses are shown in Table 1. All tobacco cultivars tested showed a strong necrotic response within the entire infiltrated area upon co-expression of matching gene pairs, while aspecific responses remained negligible. As with tobacco cv. Petite Havana SR1, transient co-expression of the *Avr9/Cf-9* gene pair in other tobacco cultivars always resulted in a more dark brown necrotic sector than co-expression of the *Avr4/Cf-4* gene pair. *Nicotiana benthamiana* and *N. plumbaginifolia* showed specific chlorotic responses, often with a necrotic centre. In *N. clevelandii,* specific necrotic responses were only visible at the site of infiltration, whereas GUS staining was also present at more distal sites. In *N. glutinosa*, specific necrosis developed within 7 days, whereas aspecific necrosis developed later. In Petunia, a specific chlorotic response developed with both gene pairs. The observation that all solanaceous species that were tested showed necrotic or chlorotic responses upon co-expression of *Avr9/Cf-9* and *Avr4/Cf-4* suggests that components that are required for *Avr/Cf*-induced responses are conserved within this family.

Transient expression of *Avr/Cf* gene pairs in the non-solanaceous species Arabidopsis, radish, lupine, pea, and flax did not induce any chlorotic or necrotic responses (Table 1), even though significant GUS staining and low aspecific responses were observed (Figure 4A). In lettuce, which showed clear GUS staining and low aspecific responses, a necrotic response was induced upon co-expression of the *Avr4/Cf-4* gene pair, whereas expression of *Avr4* and *Cf-4* in non-matching combinations with *Cf-9* and *Avr9*, respectively, did not induce necrosis (Figure 4B). Surprisingly, co-expression of the *Avr9/Cf-9* gene pair in lettuce did not result in necrosis under the conditions tested.

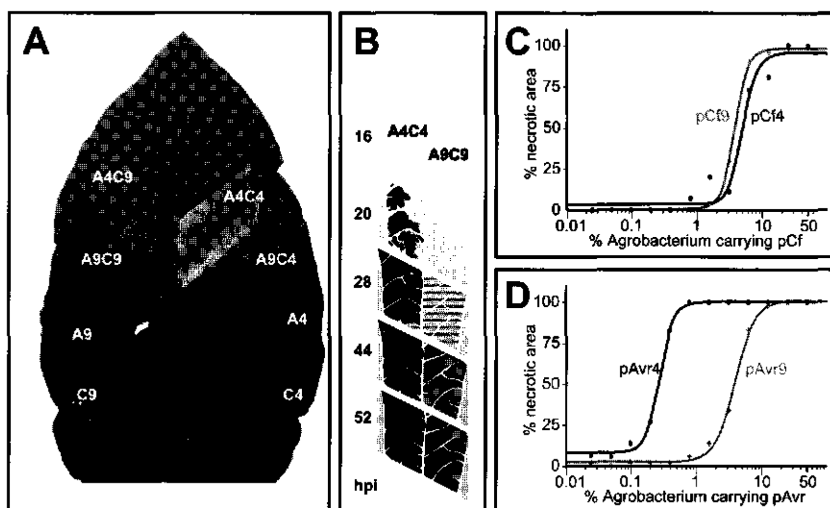

Figure 3. Comparison of necrosis induced by different matching *Avr/Cf* gene pairs.

A Transient expression and co-expression of *Avr9* (A9), *Cf-9* (C9), *Avr4* (A4), and *Cf-4* (C4) in wild-type tobacco. For transient co-expression, Agrobacterium cultures were mixed in a 1:1 ratio and infiltrated. Photograph was taken at 7 days post infiltration (dpi).

B Drawing, representing development of responses induced by co-expression of *Avr9/Cf-9* or *Avr4/Cf-4* gene pairs in wild-type tobacco. Drawings were made of the same leaf at different hours post infiltration (hpi) and show intact tissue (light grey), strong chlorotic areas (dark grey), weak chlorotic areas (grey stripes), and areas with collapsed tissue (black).

C Quantification of necrosis induced by transient *Cf* gene expression. Agrobacterium carrying pCf was diluted with Agrobacterium carrying the matching pAvr and infiltrated into wild-type tobacco leaves. pCf9 (+) and pCf4 (•) dilution series were infiltrated into opposite leaf halves. Percentage of infiltrated leaf area that had become necrotic at 7 dpi was measured and plotted against concentration of Agrobacterium that carries pCf.

D Quantification of necrosis induced by transient *Avr* gene expression. Agrobacterium carrying pAvr was diluted with Agrobacterium carrying matching pCf and infiltrated into wild-type tobacco leaves. pAvr9 (+) and pAvr4 (•) dilution series were infiltrated into opposite leaf halves. Percentage of infiltrated leaf area that had become necrotic at 7 dpi was measured and plotted against concentration of Agrobacterium that carries pAvr. **C** and **D**, One representative experiment is shown in each.

Discussion

Transient expression of *Avr/Cf* gene pairs

The gene pairs investigated in this study are derived from the fungal pathogen *C. fulvum* and its only host, tomato. As the fungus grows extracellularly, it is expected that secreted AVR proteins are perceived on the tomato plasma membrane via *R* gene products (Joosten and De Wit, 1999). By demonstrating that specific necrosis occurs upon transient expression of matching *Avr/Cf* gene pairs, we have shown for the first time that agroinfiltration can be used to study extracellular perception. Prior to this study, transient expression through agroinfiltration was only used to express the small cytoplasmic R protein Pto (Frederick et al., 1998; Rathjen et al., 1999) and to demonstrate that

Table 1. Transient expression of ß-glucuronidase (*gus*) and *Avr/Cf* gene pairs in different plant species

			Induced responses[b]		
Plant species	**Family**	**GUS[a]**	**Aspecific**	***Avr9/Cf-9***	***Avr4/Cf-4***
Nicotiana tabacum cv. Petite Havana	Solanaceae	+++	-	+++	+++
N. tabacum cv. Samsun NN	Solanaceae	+++	-	+++	+++
N. tabacum cv. Xanthi	Solanaceae	+++	-	+++	+++
N. tabacum cv. White Burley	Solanaceae	+++	-	+++	+++
N. benthamiana	Solanaceae	+++	+	++	++
N. clevelandii	Solanaceae	+++	+	+	+
N. glutinosa	Solanaceae	+++	-	+++	+++
N. rustica	Solanaceae	++	++	++	++
N. plumbaginifolia	Solanaceae	ND	-	+	+
Petunia hybrida W115	Solanaceae	ND	+	++	++
Lactuca sativa (lettuce)	Compositae	+++	-	-	+++
Arabidopsis thaliana cv. Col-0	Cruciferae	+++	-	-	-
Raphanus sativus (radish)	Cruciferae	+	-	-	-
Lupinus albus (lupine)	Leguminosae	+	-	-	-
Pisum sativum (pea)	Leguminosae	++	-	-	-
Linum usitatissimum (flax)	Linaceae	+++	-	-	-

[a] *gus* gene expression, as estimated by GUS staining at 7 days post infiltration (dpi); +, low; ++, moderate; +++, high *gus* expression; ND, not determined.

[b] Induced responses at 7 dpi by transient expression of *Avr4/Cf-9*, *Avr9/Cf-4*, or *gus* (aspecific responses), *Avr9/Cf-9* and *Avr4/Cf-4* (specific responses). -, no response compared with non-infiltrated area; +, weak chlorosis/necrosis; ++, moderate chlorosis/necrosis; +++, severe necrosis of entire infiltrated area. Co-expression was done by infiltrating cultures that were mixed in a 1:1 ratio

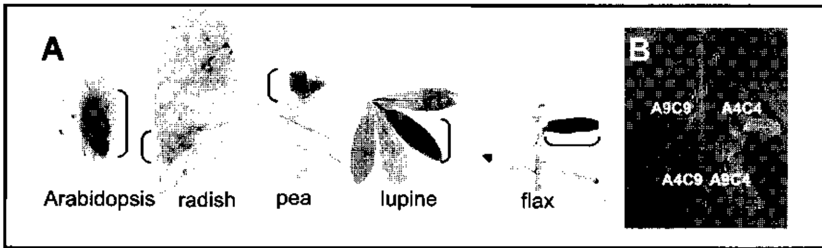

Figure 4. Expression of β-glucuronidase (*gus*) and *Avr/Cf* gene pairs in different plant species.
A Transient *gus* expression in different plant species following agroinfiltration with pGUSi. GUS staining was performed at 7 days post infiltration (dpi). The infiltrated area is indicated with a bracket.
B Transient co-expression of *Avr9* (A9), *Cf-9* (C9), *Avr4* (A4), and *Cf-4* (C4) in lettuce by infiltration of cultures that were mixed in a 1:1 ratio. Photograph was taken at 7 dpi.

perception of AvrBs3, AvrPto, Tobacco Mosaic Virus (TMV)-helicase, and PVX coat protein occurs in the cytosol (Abbink et al., 1998; Bendahmane et al., 1999; Erickson et al., 1999; Scofield et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996).

Our results demonstrate that *Cf*-mediated recognition of *Avr* gene products is retained when agroinfiltration is combined with established assays, such as transgenic tobacco plants expressing a matching gene partner, PVX-mediated *Avr* gene expression, or elicitor protein injections. Injection of a dilution series of AVR9 protein into leaves that transiently express *Cf-9* shows that agroinfiltration is sufficiently sensitive to study *Avr/Cf* interactions. Furthermore, necrosis induced by agroinfiltration of *Avr/Cf* gene pairs can be quantified and can also be used to study *Avr/Cf*-induced responses in a large number of plant species. Together, these results indicate that agroinfiltration is a powerful and versatile tool for further studies on *Avr* and *Cf* gene function.

R gene products are key components in the signal transduction pathway leading to induction of programmed cell death and defence responses that eventually stop invasion of the pathogen. Therefore, overexpression of *R* genes in the absence of matching elicitors may result in auto-necrosis. For example, overexpression of the *Pto* gene induced necrosis in the absence of AvrPto (Tang et al., 1999). The pCf constructs used in this study were all driven by a 35S promoter to ensure sufficient *Cf* gene expression. Indeed, dilution experiments showed that a culture containing only 2% pCf-carrying Agrobacterium is sufficient to induce specific necrosis in the presence of a matching *Avr* gene. Nevertheless, agroinfiltration of 35S-driven *Cf* genes in the absence of their matching *Avr* genes did not result in necrosis. This may suggest that overproduction of Cf proteins is not toxic to the plant or that mechanisms exist that keep Cf protein levels sufficiently low. pCf dilution experiments also suggest that agroinfiltration of Agrobacterium cultures that carry *Cf* genes with weak promoters should result in necrosis when expressed with their matching *Avr* genes. Indeed, agroinfiltration of the *Cf-9* gene, driven by its native promoter, was found to induce necrosis when co-expressed with *Avr9* (data not shown).

Comparison of *Avr9/Cf-9*- and *Avr4/Cf-4*-induced responses

Transient *Cf* gene expression allowed us for the first time to compare responses induced by different *Avr/Cf* gene pairs within the same leaf. We found that *Avr9/Cf-9*-induced necrosis developed slower than *Avr4/Cf-4*-induced necrosis when pAvr- and pCf-containing cultures were combined in a 1:1 ratio. However, at concentrations of pAvr-containing cultures that correspond to NC^{50} values, no temporal differences between the two gene pairs were observed. This indicates that the temporal differences between *Avr9/Cf-9*- and *Avr4/Cf-4*-induced responses are correlated with the activities of the pAvr constructs. These different pAvr activities can for example be due to differences in transcriptional or post-transcriptional modification, AVR protein stability, or perception.

Comparison of different *Avr/Cf*-induced responses in other systems has also indicated that the *Avr9/Cf-9* gene pair is less active than the *Avr4/Cf-4* gene pair (M. H. A. J. Joosten, personal communication). For example, tomato seedlings die quicker when derived from seeds of a cross between *Avr4* transgenic tomato and MM-Cf4 than when derived from seeds of a cross between *Avr9* transgenic tomato and MM-Cf9. Similarly, inoculation of PVX::*Avr4* on MM-Cf4 results in a more severe systemic necrosis than inoculation of PVX::*Avr9* on MM-Cf9 plants. Our results suggest that these temporal differences are due to differences between the *Avr9* and *Avr4* gene activities when expressed *in planta*, rather than to intrinsic differences between the functions of *Cf* gene products.

Presence of *Avr/Cf* signal transduction pathways in other plant families

The striking similarities between proteins encoded by *R* genes cloned from different plant species suggest that different *R* genes would function in other plant species. Indeed, the tomato *Pto* gene has been shown to function in *Nicotiana* spp. (Thilmony et al., 1995), the tobacco *N* gene is active in tomato (Witham et al., 1996), and the tomato *Cf-9* gene is functional in potato and tobacco (Hammond-Kosack et al., 1998). We could extend this analysis by transient expression studies and show that *Cf-9* and *Cf-4* genes from tomato function in all *Nicotiana* spp. tested, as well as in Petunia. This indicates that the signal transduction pathway required for *Avr/Cf*-mediated necrosis is conserved within the Solanaceae. It also suggests that these plant species may recruit the same signal transduction pathway to activate defence responses against their pathogens.

In contrast, Arabidopsis, radish, lupine, pea, and flax did not show necrotic responses upon transient co-expression of matching *Avr/Cf* gene pairs, even though *gus* expression was detected and background responses were sufficiently low. This indicates that these plant species lack components that are required for *Avr/Cf*-induced responses, implying that functional transfer of an *R* gene from one plant family to another has its limitations.

An exception to the above is lettuce, a composite that seems to contain all components required for *Avr4/Cf-4*-induced necrosis. This is the first report of an *R* gene that can function in a different plant family. Surprisingly, *Avr9/Cf-9*-induced necrosis was not observed in lettuce, suggesting that the level of expression of *Avr9* in lettuce is below the threshold level that is required for activity. Alternatively, lettuce may lack one or more components of the signal transduction pathway required for *Avr9/Cf-9*-induced necrosis.

Materials and Methods

Plant material, GUS staining, PVX inoculation, *and protein preparations*

Plants were grown under standard greenhouse conditions except for Arabidopsis, which was grown under short day conditions. For most assays, 4- to 8-week-old *N. tabacum* cv. Petite Havana (SR1) plants were used, unless stated otherwise. 35S::*Avr9* transgenic tobacco line SLJ6201A (Hammond-Kosack et al., 1994) and transgenic tobacco line 6A3 carrying a genomic clone of *Cf-9* were used (Kamoun et al., 1999). GUS staining was performed as described by Jefferson (1987). PVX inoculations with wild-type PVX, PVX::*Avr9*, and PVX::*Avr4* were performed as described before (Joosten et al., 1997; Hammond-Kosack et al., 1995). Synthetic AVR9 was prepared as described previously (Kooman-Gersmann et al., 1998). Apoplastic fluids were isolated from compatible *C. fulvum*-tomato interactions at 14 to 20 days after inoculation, as described by De Wit and Spikman (1982).

DNA manipulations and plasmids

All DNA manipulations were performed by standard protocols (Sambrook et al., 1989). Polymerase chain reaction (PCR) was performed with *Pfu* polymerase (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Restriction enzymes, T4 ligase, and *Escherichia coli* DH5α cells were from Life Technologies (Breda, The Netherlands). Primers were synthesised by Amersham-Pharmacia (Buckinghamshire, UK). Authenticity of all cloned PCR fragments was confirmed by sequencing.

The following plasmids were used in our studies: pFM4 and pMOG800 (Honée et al., 1998), pCf9.5 (*prp1::Cf-9*, pMOG1048; Honée et al., 1998), pGUSi (Figure 1; pMOG410; Hood et al., 1993), PVX::*Avr4* (Thomas et al., 1997), and pAvr9 (Figure 1; pMOG978; Honée et al., 1998). pFT43, containing a *Cf-4* genomic clone, was kindly provided by Frank Takken (Department of Genetics, BioCentrum Amsterdam; Takken, 1999).

pCf9 and pCf4 were constructed as follows: with *Xba*I and *Nco*I restriction sites, the 35S promoter from pFM4 was cloned into pCf9.5, thereby replacing the *prp1* promoter and creating pRH1. The 5′ part of the *Cf-4* gene was amplified from pFT43, with primers ttagtgcagccatgggttgtg and catgcaacttatttgatctcaagc (*Nco*I site is underlined). The latter primer anneals 3′ of the *Hin*dIII site, which is present in both *Cf-9* and *Cf-4*. With *Nco*I and *Hin*dIII restriction sites, the PCR product was cloned into pRH1, thereby replacing the 5′-terminal part of *Cf-9* with that of *Cf-4*, generating pRH46. The 3′ region of the *Hin*dIII restriction site of *Cf-9* and *Cf-4* genes encodes identical amino acids. The promoter-ORF-terminator cassettes of pRH1 and pRH46 were subsequently transferred to pMOG800 with *Bam*HI and *Kpn*I restriction sites, creating binary plasmids pCf9 (pRH21) and pCf4 (pRH48).

For the construction of pAvr4, the 35S promoter was amplified from pRH1 with primers gatctctagaggtcaacatggtggagcacg and aaaactgcagctcgaggtcgacaccatggtattgtaaatagtaattgtaatgttg (*Xba*I, *Pst*I, and *Nco*I sites are underlined, respectively) and cloned into pRH1 with *Xba*I and *Pst*I. This construct (pRH80) carries the 35S promoter and the PI-II terminator (An et al., 1989) flanking a multiple cloning site (*Nco*I-*Sal*I-*Xho*I-*Pst*I). The ORF encoding the mature AVR4 protein fused to the signal peptide of the pathogenesis-related gene *PR1a* was amplified from PVX::*Avr4* with primers cgttccactggagtccttttgg, ccaaaaggactccagtggaacg, ttagtgcagccatgggttgtg, and aaaactgcagtcattgcggcgtctttaccggacacg (*Nco*I and *Pst*I sites are underlined, respectively). The first two primers were designed to remove the *Pst*I site from *Avr4* by PCR overlap-extension. The PCR product was cloned into pRH80 with *Nco*I and *Pst*I, thereby creating pRH85. The promoter-ORF-terminator cassette of pRH85 was cloned into pMOG800 with *Xba*I and *Eco*RI, creating the binary plasmid pAvr4 (pRH87).

Agrobacterium-mediated transient expression

The *Agrobacterium tumefaciens* strain MOG101 (Hood et al., 1993) was transformed by electroporation. Recombinant Agrobacterium containing the different binary plasmids was grown overnight (28°C, 200 rpm; LABOTECH RS500; Labotec, Belgium) in tubes containing 3 ml of YEB medium (per litre: 5 g of beef extract [Sigma, St. Louis, MO], 1 g of yeast extract [Oxoid, Hampshire, UK], 5 g of bacteriological peptone [Oxoid], 5 g of sucrose, and 2 ml of 1 M $MgSO_4$) containing 50 µg of kanamycin (Duchefa, Haarlem, The Netherlands) per ml and 25 µg of rifampicin (Sigma) per ml. These cultures were used to inoculate a 300-ml conical flask containing 100 ml of YEB medium supplemented with 1 ml of 1 M *N*-morpholino-ethanesulfonic acid (MES; Sigma), 50 µg of kanamycin per ml, and 2 mM acetosyringone (Aldrich, Steinheim, Germany). After overnight incubation (28°C, 200 rpm; LABOTECH RS500; Labotec, Belgium), cells were harvested at an OD_{600} of 0.6 to 1.2 by centrifugation (8′, 4,000 x *g*) and resuspended in MMA to a final OD of 2 (1 litre of MMA: 5 g of MS salts [Duchefa], 1.95 g of MES, 20 g of sucrose, pH adjusted to 5.6 with 1 M NaOH), containing 200 µM acetosyringone. At this stage, cultures were mixed as described in the figure legends. Cultures were infiltrated into leaves with a 2-ml disposable syringe without a needle. Leaves were superficially wounded with a needle to improve infiltration.

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Chapter 3

Identification of Distinct Specificity Determinants in Resistance Protein Cf-4 Allows Construction of a Cf-9 Mutant That Confers Recognition of Avirulence Protein AVR4

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Abstract

The tomato resistance genes *Cf-4* and *Cf-9* confer specific, hypersensitive response–associated recognition of *Cladosporium fulvum* carrying the avirulence genes *Avr4* and *Avr9*, respectively. *Cf-4* and *Cf-9* encode type I transmembrane proteins with extracellular leucine-rich repeats (LRRs). Compared with Cf-9, Cf-4 lacks two LRRs and differs in 78 amino acid residues. To investigate the relevance of these differences for specificity, we exchanged domains between Cf-4 and Cf-9, and mutant constructs were tested for mediating the hypersensitive response by transient co-expression with either *Avr4* or *Avr9*. We show that the number of LRRs is essential for both Cf-4 and Cf-9 function. In addition, Cf-9 specificity resides entirely in the LRR domain and appears to be distributed over several distant LRRs. In contrast, Cf-4 specificity determinants reside in the N-terminal LRR-flanking domain and three amino acid residues in LRRs 13, 14, and 16. These residues are present at putative solvent-exposed positions, and all are required for full Cf-4 function. Finally, we show that Cf-9 carrying the specificity determinants of Cf-4 has recognitional specificity for AVR4. The data indicate that diversifying selection of solvent-exposed residues has been a more important factor in the generation of Cf-4 specificity than has sequence exchange between *Cf-4* progenitor genes. The fact that most variant residues in Cf-4 are not essential for Cf-4 specificity indicates that the diverse decoration of R proteins is not fully adapted to confer recognition of a certain avirulence determinant but likely provides a basis for a versatile, adaptive recognition system.

Introduction

Because plants are subjected to environments that change continuously, they are armed with recognition systems that can sense diverse biotic and abiotic stresses and subsequently mediate the induction of appropriate responses. A recognition system that can deal with various pathogens is especially crucial for the survival of the plant. Specific recognition of invading pathogens frequently is mediated by resistance (*R*) genes. Upon recognition of the matching pathogen-derived avirulence determinant (AVR), various defence responses are triggered, often including a hypersensitive response. The hypersensitive response involves the death of the tissue surrounding the primary infection site and thereby restricts further growth of the invading pathogen (Hammond-Kosack and Jones, 1996).

Surprisingly, *R* genes that confer resistance to different types of pathogens encode very similar proteins, indicating that in plants, flexible recognition systems are used to monitor

attacks by a diverse array of pathogens. The largest class of *R* genes encodes proteins that are likely located in the cytoplasm and contain leucine-rich repeats (LRRs) and a nucleotide-binding site (NBS). Members of this NBS-LRR class have been cloned from various plant species and confer race-specific resistance against viruses, bacteria, fungi, oomycetes, nematodes, or insects (reviewed in Van der Biezen and Jones, 1998). Some *R* genes encode proteins that are likely located in the plasma membrane and carry extracellular LRRs. The *Xa21* gene from rice encodes a receptor-like kinase containing a cytoplasmic kinase and an extracellular LRR domain (Song et al., 1995). The products that are encoded by $Hs1^{Pro\text{-}1}$ from sugar beet and the *Cf* genes from tomato lack the cytoplasmic kinase domain and predominantly consist of extracellular LRRs (Jones et al., 1994; Dixon et al., 1996, 1998; Cai et al., 1997; Thomas et al., 1997; Takken et al., 1999).

Because pathogens continuously attempt to circumvent recognition by the host, the plant recognition system must be able to generate new specificities. Mechanisms by which *R* genes with new specificities evolve include sequence exchange between homologous genes and selective mutations of solvent-exposed amino acid residues (Parniske et al., 1997; Song et al., 1997; Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Ellis et al., 1999; Noël et al., 1999; Parniske and Jones, 1999; Bittner-Eddy et al., 2000).

Specific recognition mediated by LRR proteins also plays a role in other plant processes. For example, many different receptor-like kinases with extracellular LRRs are involved in plant development. *ERECTA* is required for proper organ elongation, *CLAVATA-1* determines cell fate in shoot and floral meristems, and *BRI1* encodes a putative brassinosteroid receptor (Torii et al., 1996; Clark et al., 1997; Li and Chory, 1997). Polygalacturonase-inhibiting proteins have a role in plant defence and are extracellular LRR proteins that bind specifically, and consequently inhibit, fungal endopolygalacturonases (De Lorenzo and Cervone, 1997).

LRRs are highly specialised protein binding motifs that are also present in various proteins of animals and bacteria (reviewed in Buchanan and Gay, 1996). Within a single LRR module, the xxLxLxx motif folds as a β-sheet in which the leucine residues (L) form a hydrophobic core, whereas the side chains of the flanking amino acid residues (x) are solvent exposed. In multiple LRRs, the β-sheets are aligned in parallel and form a surface decorated with solvent-exposed residues that can interact specifically with a ligand. Significant progress in the understanding of protein–protein interactions involving LRR proteins comes from crystallographic studies of ribonucleases and LRR-containing ribonuclease inhibitors (RIs). Co-crystallisation of porcine RI and human RI (hRI) with the enzymes RNaseA and angiogenin, respectively, revealed that of the 28 amino acid residues of porcine RI that interact with RNaseA, 26 are at solvent-exposed positions (Kobe and Deisenhofer, 1996). Similarly, of the 26 amino acid residues of hRI that interact with angiogenin, 25 are at solvent-exposed positions (Papageorgiou et al., 1997). Likewise, it can be expected that solvent-exposed amino acid residues of LRRs in R proteins are involved in interactions with matching AVR proteins. Consistent with this theory, it was found that R proteins with specificity for different avirulence determinants differ predominantly in amino acid residues at putative solvent-exposed positions (Parniske et al., 1997; Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Noël et al., 1999; Bittner-Eddy et al., 2000). This finding indicates that these variant amino acid residues are present to adapt the decoration of R proteins to optimal recognition of the matching AVR determinants.

To understand or even modify the specificity of R proteins, studies on domains that determine their specificity are of great interest. For such studies, two functional homologous R proteins with different specificity are required. The *R* genes *Cf-4* and *Cf-9* from tomato confer resistance to strains of the biotrophic leaf mold fungus *C. fulvum* that carry the avirulence genes *Avr4* and *Avr9*, respectively (reviewed in Joosten and De Wit, 1999). Both *Avr* genes encode stable elicitor proteins that are secreted into the apoplast of tomato leaves. Apart from being small and cysteine rich, the avirulence determinants AVR4 and AVR9 have no sequence similarities. In contrast, the matching *R* genes encode proteins that share 91% amino acid residue identity (Thomas et al., 1997). The primary structures and the alignment of Cf-4 and Cf-9 are shown in Figure 1. Compared with Cf-9, Cf-4 lacks 10 amino acid residues in the B-domain, one residue in LRR 4, and two complete LRRs at the position where Cf-9 has LRRs 11 and 12. Cf-4 further differs from Cf-9 by 67 amino acid residues that are confined to the N-terminal half of the proteins. Six and four variant residues reside in the signal peptide (A-domain) and B-domain, respectively, whereas the remaining 57 variant amino acid residues are present in the LRRs, of which 32 are located at putative solvent-exposed positions of the β-sheets (Figure 1).

It must be stressed that no evidence exists for a direct interaction between AVR and Cf proteins. It is likely that AVR9 is perceived by plants carrying *Cf-9* through a high-affinity binding site for AVR9 that was identified in plasma membranes of solanaceous plants (Kooman-Gersmann et al., 1996, 1998). A similar situation is possible for the perception of AVR4. Although the exact nature of the "ligands" of Cf proteins is unknown, Cf proteins are expected to be involved in specific interactions, possibly through the solvent-exposed amino acid residues in the LRR domain.

The high homology between Cf-4 and Cf-9 proteins provides an excellent opportunity to determine which domains of the Cf proteins confer specificity. Because Cf and AVR function are retained upon Agrobacterium-mediated transient expression in tobacco (chapter 2), we used this technique as a quick and versatile tool to study Cf specificity. In this study, we show that the functions of both Cf-9 and Cf-4 are strongly affected when the number of LRRs is changed. Moreover, the specificity of Cf-9 resides entirely in the LRRs and appears to be distributed over several distant LRRs. In contrast, the specificity of Cf-4 was found to reside in the B-domain and three Cf-4–specific amino acid residues. These residues are present at solvent-exposed positions within LRRs 13, 14, and 16 and collectively contribute to Cf-4 specificity. Introduction of these specificity determinants into the LRRs of Cf-9 results in recognitional specificity for AVR4 instead of AVR9. Our results indicate that diversifying selection of solvent-exposed amino acid residues was a more important factor in the generation of Cf-4 specificity than was sequence exchange between *Cf-4* progenitor genes. The fact that most variant amino acid residues in Cf-4 are not essential for Cf-4 specificity indicates that decoration of R proteins is not fully adapted to confer recognition of a certain avirulence determinant but likely provides a basis for a versatile, adaptive recognition system.

Results

The LRR domain of Cf proteins is required for specificity

The differences between Cf-4 and Cf-9 suggest that the LRR domain determines specificity for mediating recognition of AVR4 and AVR9, respectively. To test this notion, we exchanged

the LRRs of Cf-4 with those of Cf-9. This exchange was facilitated by the introduction of *Cla*I, *Sal*I, and *Bam*HI restriction sites into the *Cf-4* and *Cf-9* genes (Figure 1). Only introduction of the *Cla*I site resulted in a substitution of two amino acid residues (V83I and H84D) in the B-domain of both Cf-4 and Cf-9. Mutants were assayed through transient co-expression with *Avr4* or *Avr9* by agroinfiltration of tobacco leaves (see material and methods). This assay demonstrated that introduction of the restriction sites did not affect *Cf* gene function (Figure 2A, mutants 127 and 109). Exchange of all LRRs of Cf-4 with those of Cf-9 abolished Cf-4 function in our assays (Figure 2A, mutant 37), indicating that the LRRs of Cf-4 are required for Cf-4 specificity. Because mutant 37 had gained Cf-9 function, we concluded that specificity for AVR9 recognition resides only in the LRRs of Cf-9.

Cf-4 specificity also resides in the B-domain

To examine whether the LRR domain of Cf-4 is sufficient to confer AVR4 responsiveness, we exchanged all LRRs of Cf-9 with those of Cf-4. Surprisingly, this mutant showed significantly reduced Cf-4 activity (Figure 2B, mutant 337), indicating that the A- and/or B-domains of Cf-4 are required for Cf-4 specificity. The A-domain, which has been suggested to function as a signal peptide for extracellular targeting (Jones et al., 1994), is cleaved off in the mature Cf protein (Luderer et al., 2001). Thus, in mature proteins, mutant 337 differs from fully functional Cf-4 by only three amino acid residues and a deletion of 10 amino acid residues in the B-domain (compare mutants 127 and 337 in Figures 2A and 2B, respectively). To determine which of these features are required for Cf-4 function, we constructed mutants that contained either the Cf-4–specific amino acid residues or the Cf-4–specific deletion in the B-domain (Figure 2B, mutants 352 and 353, respectively). Both mutants showed reduced activity compared with wild-type Cf-4, indicating that both features contribute significantly to Cf-4 specificity. In addition, in five replicate experiments, we observed that mutant 353 was more active than mutant 352, indicating that within the B-domain of Cf-4 the deletion of 10 amino acid residues is more important for Cf-4 specificity than the three Cf-4–specific amino acid residues.

The number of LRRs strongly affects Cf-4 and Cf-9 function

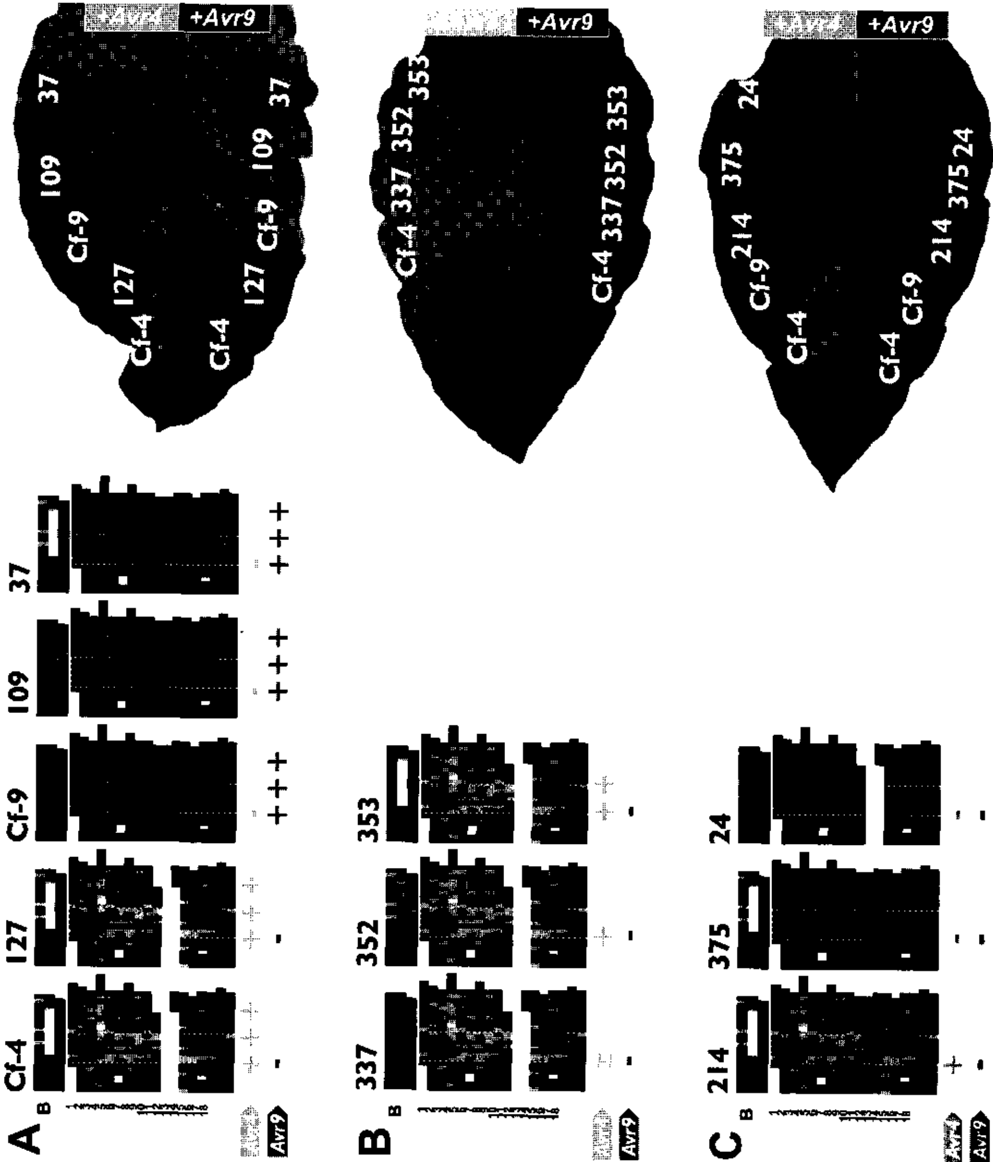
The LRR domain of Cf-4 lacks LRRs 11 and 12 compared with Cf-9 (Figure 1). To examine the role of this deletion in Cf-4 function, we inserted LRRs 11 and 12 of Cf-9 into Cf-4. This mutant showed a significant reduction in activity compared with wild-type Cf-4 (Figure 2C, mutant 214), indicating that this Cf-4–specific deletion is important for Cf-4 function. To determine whether deletion of LRRs 11 and 12 in the LRR domain of Cf-9 is sufficient to confer Cf-4 specificity, we deleted LRRs 11 and 12 of Cf-9 from mutant 37, which already contained the B-domain of Cf-4 (Figure 2A). This mutant was not responsive to AVR4 (Figure 2C, mutant 375), suggesting that in addition to the B-domain and the deletion of LRRs 11 and 12, Cf-4–specific amino acid residues within

Figure 1. Primary structure and alignment of Cf-4 and Cf-9 resistance proteins.
Amino acid residues of Cf-4 and Cf-9 that are identical are shown in normal script. Cf-4– and Cf-9–specific residues are shown in bold at top and bottom line, respectively. The numbering (left) corresponds to the Cf-9 protein sequence. Potential *N*-glycosylation sites (NxS/T) in Cf-4 and Cf-9 are overlined and underlined, respectively. The box indicates the various β-sheets (consensus xxLxLxx), each of which contains five solvent-exposed amino acid

```
SP   1   MDCVKLVFLMLYTFLCQLALSSS

B    24  LPHLCPEDQALSLLQFKNMFTIN
     47  PNASDYCYDIRTYVDIQSYPRTL
     70  SWNKSTSCCSWDGVHCDETTGQ
                      ID
                xxLxLxx
1    92        VIALDLRCSQLQGKFHSNSS
2    112 LFQLSNLKRLDLSFNNFTGSLISPK
3    136 FGEFSNLTHLDLSHSSFTGLIPSE
4    161 ICHLSKLHVLRICDQYGLSLVPYNFELL
5    189 LKNLTQLRELNLESVNISSTIPSN
6    213 FS--SHLTTLQLSGTELHGILPER
7    235 VFHLSNLQSLHLSVNPQLTVRFPTTK
8    261 WNSSASLMTLYVDSVNIADRIPKS
9    285 FSHLTSLHELYMGRCNLSGPIPKP
10   309 LWNLTNIVFLHLGDNHLEGPISH
11   332 FTIFEKLKRLSLVNNNFDGGLEF
12   355 LSFNTQLERLDLSSNSLTGPIPSN
13   379 ISGLQNLECLYLSSNHLNGSIPSW
14   403 IFSLPSLVELDLSNNTFSGKIQE
15   426 FKS-KTLSAVTLKQNKLKGRIPNS
16   449 LLNQKNLQLLLLSHNNISGHISSA
17   473 ICNLKTLILLDLGSNNLEGTIPQCV
18   498 VERNEYLSHLDLSKNRLSGTINTT
19   522 FSVGNILRVISLHGNKLTGKVPRS
20   546 MINCKYLTLLDLGNNMLNDTFPNW
21   570 LGYLFQLKILSLRSNKLHGPIKSSGN
22   596 TNLFMGLQILDLSSNGFSGNLPERI
23   621 LGNLQTMKEID
     632 ESTGFPEYISDPYDIYYNYLTTISTKGQDYDS
24   664 VRILDSNMIINLSKNRFEGHIPSI
25   688 IGDLVGLRTLNLSHNVLEGHIPAS
26   712 FQNLSVLESLDLSSNKISGEIPQQ
27   736 LASLTFLEVLNLSHNHLVGCIPKG
D    760 KQFDSFGNTSYQGNDGLRGFPLSKLCGG
E    788 EDQVTTPAELDQEEEEEDSPMISWQ
TM   806 GVLVGYGCGLVIGLSVIYIMWSTQYPAWFS
G    843 RMDLKLEHIITTKMKKHKKRY
                              COOH
```

residues (x). Residues encoded at restriction sites are indicated with grey boxes. Introduction of a *Cla*I site resulted in two residue changes, whereas the *Sal*I and *Bam*HI sites were introduced as silent mutations. The *Eco*RI site is endogenous for *Cf-4* and *Cf-9*. Domains, indicated at left, are as follows: SP, signal peptide (A-domain); B, cysteine-rich domain; 1 to 27, LRRs; D, domain without conspicuous features; E, acidic do-main; TM, putative transmembrane domain; G, basic domain, representing the putative cytoplasmic tail.


A
Cf-4
127
Cf-9
109
37
Avr9
B
337
352
353
C
214
375
24
Avr9
+Avr9
Cf-4
127
Cf-9
109
37
+Avr9
Cf-4 337 352 353
+Avr9
Cf-4
Cf-9
214
375
24

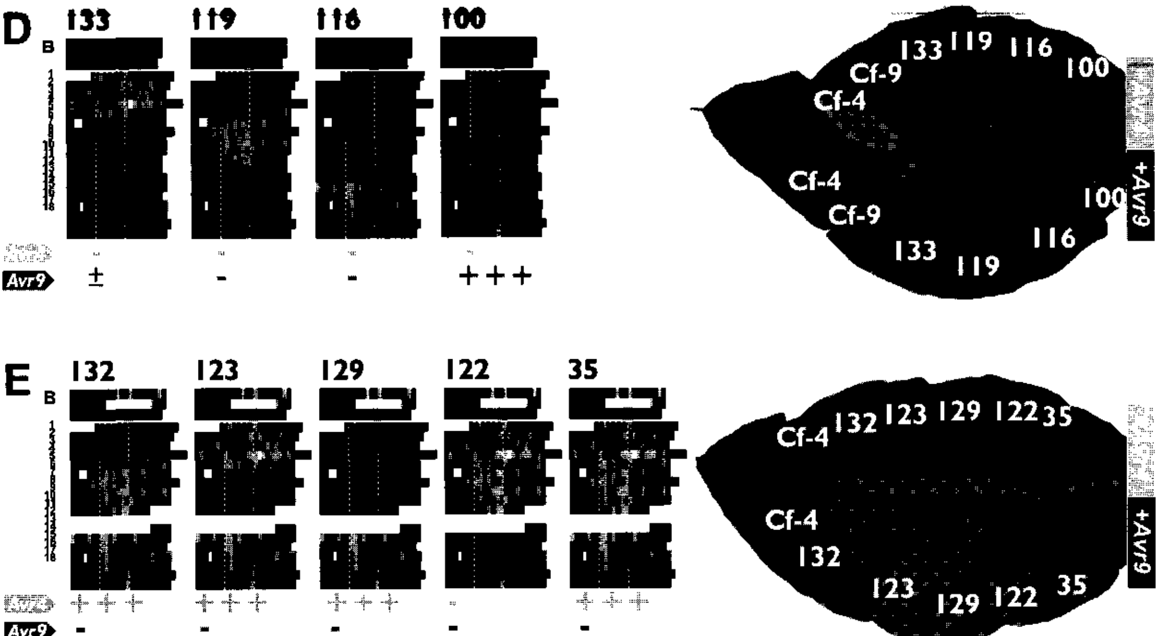


Figure 2. Domain swap analysis reveals distinct specificity determinants in Cf-4. Amino acid residues of the Cf-4 and Cf-9 proteins are represented as blocks that make up the different protein domains (indicated at left; see also Figure 1). Only parts of the mature proteins that contain variant residues are shown. Amino acid residues that are identical are shown in black, whereas Cf-4– and Cf-9–specific residues are shown in light and dark grey, respectively. Cf-4–specific deletions are shown in white. Positions corresponding to restriction sites are indicated with squares. The numbers of the mutants are indicated above the blocks. Below the blocks, the *Avr* responsiveness of the mutant is indicated compared with wild-type Cf-4 (light grey, top) or Cf-9 (dark grey, bottom): -, no necrosis above background; +, incomplete necrosis of infiltrated area (10 to 50%); ++, almost complete necrosis of infiltrated area (50 to 100%); +++, as active as wild-type Cf. The tobacco leaves shown at right illustrate responses observed upon transient co-expression of (mutated) *Cf* genes with *Avr4* (top half of the leaf) or *Avr9* (bottom half of the leaf). Photographs were taken 7 days after infiltration and are representative of at least three independent experiments. Colour differences of necrotic sectors are due to differences in leaf age and result from the fact that *Avr9* is less active than *Avr4* when expressed *in planta* (see chapter 2).

A The LRR domain of Cf proteins is required for specificity.
B Cf-4 specificity also resides in the B-domain.
C The number or LRRs strongly affects Cf-4 and Cf-9 function.
D Cf-9 specificity appears to be distributed over several distant LRRs.
E Cf-4–specific residues in LRRs 13 to 16 are essential for Cf-4 function.

other LRRs are required for Cf-4 function. Interestingly, mutant 375 also was not active in AVR9 recognition, indicating that deletion of LRRs 11 and 12 from Cf-9 abolishes Cf-9 function (compare with mutant 37, Figure 2A). Indeed, deletion of LRRs 11 and 12 from wild-type Cf-9 protein was sufficient to abolish Cf-9 function in our assays (Figure 2C, mutant 24).

Cf-9 specificity is likely distributed over several distant LRRs

In addition to the presence of LRRs 11 and 12, the LRRs of Cf-9 differ from those of Cf-4 in 57 amino acid residues. To define the region within the LRR domain that specifies Cf-9 function, we exchanged the LRRs of Cf-9 with those of Cf-4 in blocks of five LRRs. Replacement of LRRs 1 to 5 significantly reduced Cf-9 function, whereas exchange of LRRs 6 to 10 or LRRs 13 to 16 abolished Cf-9 function in our assays (Figure 2D, mutants 133, 119, and 116, respectively). This finding indicates that Cf-9–specific amino acid residues that are required for Cf-9 function are distributed over several distant LRRs. The presence of one Cf-4–specific amino acid, N511, in LRR 18 of Cf-9 did not affect Cf-9 function (Figure 2D, mutant 100).

Cf-4–specific amino acid residues residing in LRRs 13 to 16 are essential for Cf-4 function

To define the region within the LRR domain that specifies Cf-4 function, the LRRs of Cf-4 were exchanged with those of Cf-9 in blocks of five LRRs. Surprisingly, replacement of LRRs 1 to 5 or LRRs 6 to 10 of Cf-4 with those of Cf-9 did not affect Cf-4 function (Figure 2E, mutants 132 and 123, respectively), indicating that the Cf-4–specific amino acid residues in LRRs 1 to 10 of Cf-4 are not essential for Cf-4 function. Indeed, a Cf-4 mutant containing LRRs 1 to 10 of Cf-9 retained complete Cf-4 function (Figure 2E, mutant 129). In contrast, replacement of LRRs 13 to 16 of Cf-4 with those of Cf-9 abolished Cf-4 function completely in our assays (Figure 2E, mutant 122), indicating that Cf-4–specific amino acid residues within LRRs 13 to 16 are required for Cf-4 function. Finally, the presence of one Cf-9–specific amino acid residue, K511, in LRR 18 of Cf-4 did not affect Cf-4 function (Figure 2E, mutant 35).

Within LRRs 13 to 16, residues W389, G411, and F457 all are required for full Cf-4 function

Having shown that only replacement of LRRs 13 to 16 of Cf-4 with those of Cf-9 abolishes Cf-4 function in our assays, we studied the roles of the remaining 11 variant amino acid residues within these LRRs (Figure 3A) in Cf-4 function in more detail. To facilitate the exchange of individual amino acid residues within LRRs 13 to 16, we introduced an *Xho*I restriction site as a silent mutation at a position in the open reading frame corresponding to LRR 13 (Figure 3A). Introduction of this restriction site did not alter Cf-4 function (Figure 3B, mutant 229). Again, substitution of all variant amino acid residues with Cf-9–specific residues abolished Cf-4 function in our assays (Figure 3B, mutant 194). Exchanging the first five Cf-4–specific amino acid residues in LRR 13 with those of Cf-9 reduced Cf-4 function slightly (Figure 3B, mutant 230). Quantitative comparison of this mutant with wild-type Cf-4 revealed only a minor difference in Cf-4 activity (Figure 3B, curve a). This finding suggests that Cf-4 specificity resides mainly in the remaining six Cf-4–specific residues: N394, I410, G411, T433, P444, and F457. Indeed, a Cf-4 mutant in which these six residues were replaced with those of Cf-9 was inactive in our assays (Figure 3B, mutant 193).

To identify which of the six Cf-4–specific amino acid residues are essential for Cf-4 function, Cf-4 mutants were generated in which each of the six amino acid residues was replaced individually with a Cf-9–specific amino acid residue (Figure 3B, mutants 256, 254, 247, 246, 248, and 220). Surprisingly, all single mutants retained Cf-4 function, albeit not all at the same level. Mutants carrying N394H, I410V, T433A, or P444R substitutions fully retained Cf-4 function (Figure 3B, mutants 256, 254, 246, and 248, respectively), whereas mutants carrying G411E or F457L substitutions showed reduced Cf-4 activity (Figure 3B, mutants 247 [curve b] and 220 [curve c], respectively). Significantly, a *Cf-4* double mutant with both G411E and F457L substitutions was inactive in our assays (Figure 3B, mutant 264), suggesting that the G411E and F457L substitutions collectively disrupt Cf-4 function. Moreover, simultaneous introduction of the N394H, I410V, T433A, and P444R substitutions did not affect Cf-4 function (Figure 3B, mutant 257, curve d). Thus, within the last six Cf-4–specific amino acid residues present in LRRs 13 to 16, only G411 and F457 are essential for full Cf-4 function.

Having identified the two Cf-4–specific amino acid residues that are required in the last six variant positions in LRRs 13 to 16, we focused on the importance for Cf-4 function of the residues at the first five variant positions of LRRs 13 to 16. Again, a mutant of Cf-4 carrying Cf-9–specific amino acid residues except G411 and F457 showed reduced Cf-4 activity (Figure 3B, mutant 263, curve e), consistent with the reduction in activity of mutant 230 (Figure 3B, curve a). The presence of an essential Cf-4–specific residue in the first five positions became more evident from a comparison based on Cf-4 mutants that carried either F457 or G411 in the last six variant positions (Figure 3B, mutants 216 and 275, respectively). In both cases, Cf-4 function was abolished completely in our assays by introducing Cf-9–specific residues in the first five positions (Figure 3B, mutants 265 and 266, respectively). Therefore, the inactive mutants carrying only F457 (mutant 265) or G411 (mutant 266) were used in a gain-of-function approach to determine which of the first five variant amino acid residues in LRR 13 is/are important for Cf-4 function.

Because we had shown that within the last six variant amino acid residues, only residues at putative solvent-exposed positions are important for Cf-4 function, we focused on amino acid residues Q386, I387, and W389, which are present at putative solvent-exposed positions of the β-sheets of LRR 13 (Figure 3A). We inserted each of these residues separately into the inactive mutants 265 and 266. Combination of amino acid residue F457 with residue Q386 or I387 did not restore Cf-4 function (Figure 3B, mutants 354 and 336, respectively), whereas combination with residue W389 restored Cf-4 activity (Figure 3B, mutant 335, curve f) to a level comparable to that of mutant 216, which carries all Cf-4–specific amino acid residues in the first five variant positions of LRR 13. Similarly, combination of amino acid residue G411 with residue Q386 or I387 did not restore Cf-4 function (Figure 3B, mutants 355 and 333, respectively), whereas combination with residue W389 restored Cf-4 activity (Figure 3B, mutant 331, curve g) to a level comparable to that of mutant 275. These data demonstrate that within LRR 13, only the Cf-4–specific amino acid residue W389 contributes to Cf-4 function.

To establish whether amino acid residues W389, G411, and F457 are sufficient to confer Cf-4 function, they were introduced simultaneously into Cf-4 mutant 194, which carries LRRs 13 to 16 of Cf-9 (Figure 3B). Introduction of the three Cf-4–specific amino acid residues into these LRRs restored Cf-4 function completely (Figure 3B, mutant 332, curve h), indicating that within LRRs 13 to 16 of Cf-4, presence of amino acid residues W389, G411, and F457 are required and sufficient for full Cf-4 function.

Quantitative analysis of the gain-of-function mutants (Figure 3B, curves f and g) also confirmed the relative importance of the substitutions observed in the loss-of-function approach (Figure 3B, curves a to c). The W389Y substitution resulted in a two-fold reduction in Cf-4 activity compared with wild-type Cf-4 (Figure 3B, curves a and e), whereas the G411E substitution resulted in an eight- to 16-fold reduction in Cf-4 activity (Figure 3B, curves b and f). The F457L substitution resulted in two- to threefold reduced Cf-4 activity (Figure 3B, curves c and g). Thus, the relative importance of these substitutions for the attenuation of Cf-4 function is G411E ≥ F457L > W389Y.

Within the LRRs of Cf-9, deletion of LRRs 11 and 12 and introduction of W389, G411, and F457 are sufficient to confer Cf-4 function

To determine whether deletion of LRRs 11 and 12, together with introduction of the amino acid residues W389, G411, and F457 into the LRRs of Cf-9, is sufficient to confer Cf-4 function, such a mutant was generated from mutant 37, which was fully responsive to AVR9 (Figures 2A and 4). Although slightly reduced compared with that of wild-type Cf-4, this mutant clearly showed specificity for AVR4, whereas Cf-9 function was lost completely (Figure 4, mutant 367). Thus, within the LRRs of Cf-9, deletion of LRRs 11 and 12 and introduction of amino acid residues W389, G411, and F457 are sufficient to confer Cf-4 function.

Discussion

Because many *R* genes that confer resistance to a variety of pathogens appear to encode homologous proteins, it is of great interest to discover the features of R proteins that determine specificity for avirulence determinants of pathogens. Reports on dissecting the domains that determine specificity in R proteins have been limited, because at least two homologous proteins with different specificities are required to address this question. The distinct specificity and high similarity of Cf-4 and Cf-9, together with the availability of the matching avirulence genes *Avr4* and *Avr9*, respectively, provided a unique opportunity to identify domains within both R proteins that determine specificity. Using agroinfiltration, we found that the number of LRRs is essential for both Cf-4 and Cf-9 function. In addition, we showed that Cf-9 specificity resides entirely in the LRR domain and is likely distributed over several distant LRRs. In contrast, Cf-4 specificity resides in the B-domain and three amino acid residues present at putative solvent-exposed positions of LRRs 13 to 16. Finally, a Cf-9 mutant carrying the features that are required for Cf-4 specificity confers recognition of AVR4.

The domain swap analysis of the tomato *Cf* resistance genes presented was based on a functional assay in tobacco. We cannot exclude that mutant *Cf* genes behave somewhat different in tomato and tobacco. Additional proteins that are required for AVR perception and interact with the Cf protein might differ slightly between tomato and tobacco. Thus far, however, we found that AVR9 and AVR4 perception occurs with the same specificity in tomato and tobacco (chapter 2), suggesting that the tomato *Cf* genes function similarly both in tomato and tobacco. We also cannot exclude that different Cf mutants differ in protein stability, because we did not examine protein accumulation of the mutants. However, because we exchanged domains between functional proteins that occur in nature, destabilisation of proteins is not likely to occur.

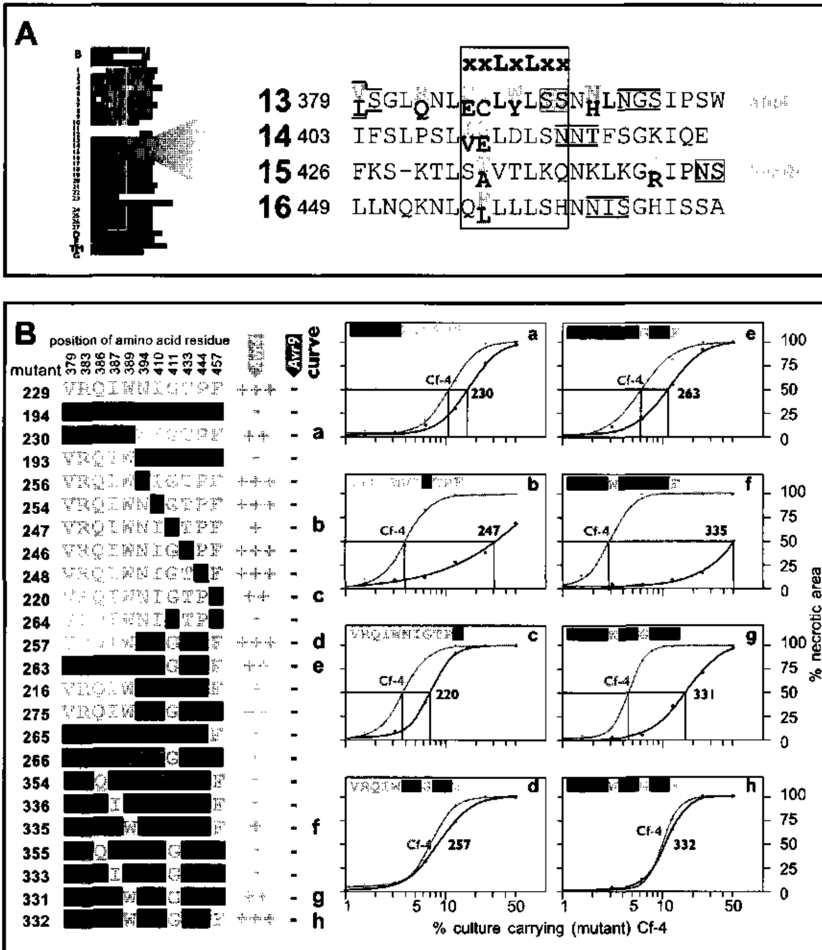


Figure 3. Three amino acid residues within LRRs 13 to 16 contribute to Cf-4 function.

A Detailed representation of LRRs 13 to 16. All mutations shown in Figure 3B were introduced into the Cf-4 backbone (left). The enlargement shows an alignment of LRRs 13 to 16 of Cf-4 and Cf-9. Variant amino acid residues between Cf-4 and Cf-9 are shown in light and dark grey, respectively. Additional details are described in Figure 1.

B Identification of single amino acid residues W389, G411, and F457 that contribute to Cf-4 function. Only amino acid residues that differ between Cf-4 and Cf-9 within LRRs 13 to 16 are shown. All mutations were present in the Cf-4 backbone (Figure 3A). Cf-4–specific amino acid residues are shown in light grey, and Cf-9–specific amino acid residues are boxed and shown in dark grey. The activity of the various Cf-4 mutants, upon co-expression with either *Avr4* or *Avr9*, is indicated at right: -, no necrosis; +, incomplete necrosis; ++ and +++, complete necrosis. Discrimination between ++ and +++ was determined by quantitative comparisons (curves a to h). For quantitative comparisons, Agrobacterium cultures that carry a plasmid that encodes a (mutant) Cf-4 protein were mixed in different ratios with a culture of equal density carrying an AVR4-encoding plasmid and infiltrated into opposite tobacco leaf halves. At 7 days after infiltration, the percentage of infiltrated area that had become necrotic was measured and plotted against the percentage of culture carrying (mutant) Cf-4.

The B-domain contributes to specificity in Cf proteins

The presumed binding capacities of the LRR domain, especially the large variation in solvent-exposed amino acid residues in this domain, suggest that the LRRs determine the specificity of Cf proteins. However, we have shown that the B-domain contributes to Cf-4 specificity. This finding suggests that in Cf-4, the B-domain is required for interaction with a component that is not essential for Cf-9 function. Especially the 10–amino acid residue deletion in the B-domain is important for Cf-4 function. It is striking that this region in the B-domain of Cf homologs is hypervariable for deletions and amino acid residue substitutions (Parniske et al., 1997; Parniske and Jones, 1999), suggesting that it generally contributes to specificity in Cf proteins. However, in contrast with Cf-4 function, Cf-9 function is not affected when its B-domain is replaced with that of Cf-4. Thus, at this stage, it is difficult to predict whether the requirement of a particular B-domain for Cf specificity is an exception rather than a rule. Also, for R proteins of the NBS-LRR class, specificity can reside outside the LRR domain, as has been found in flax, in which the L6 and L7 proteins vary only outside the LRR domain and yet have different specificities (Ellis et al., 1999).

Importance of the Number of LRRs

Among the 15 Cf proteins that are homologous with Cf-4 and Cf-9, Cf-4 is exceptional in having 25 rather than 27 LRRs (Parniske et al., 1997; Parniske and Jones, 1999). In this report, we show that this feature is important for Cf-4 function. We also show that Cf-9 function is abolished in our assays when LRRs 11 and 12 are deleted. As a consequence, it is unlikely that a hybrid Cf protein with recognitional specificities for both AVR4 and AVR9 can be constructed.

Variation in the number of LRRs is a general feature of R proteins. Cf proteins that show homology with Cf-2 carry 25, 26, 31, 32, 34, or 38 LRRs (Dixon et al., 1998), whereas NBS-LRR proteins encoded by the RPP5 locus carry 13, 17, 21, 23, or 25 LRRs (Noël et al., 1999). However, the importance of the number of LRRs for R protein function has been reported in only a few cases. The non-functional *Cf-5* homolog *Hcr2-5D* encodes a protein that differs from the functional Cf-5 protein by having two additional LRRs (Dixon et al., 1998). Similarly, inactive alleles of the *M* gene from flax encode NBS-LRR proteins with a reduced number of LRRs (Anderson et al., 1997), and an inactive *RPP5* allele from Arabidopsis encodes an NBS-LRR protein with a duplication of four LRRs (Parker et al., 1997).

Studies of the interaction between hRI and angiogenin showed that LRRs of hRI have multiple interaction points with angiogenin (Papageorgiou et al., 1997). The 26 amino acid residues of hRI that interact with angiogenin are scattered throughout the 13 LRRs of hRI. Deletion of one or more LRRs abolished the hRI–angiogenin interaction, an observation that could not be explained solely by the loss of the few interacting amino acid residues within the deleted LRRs. This finding suggests that deletion of LRRs can result in loss of interaction with the remaining LRRs or can significantly change the overall three-dimensional structure of the LRR protein. Similar strong structural effects can explain the requirement of a specific number of LRRs in Cf-4, Cf-9, and other R proteins.

The role of putative solvent-exposed amino acid residues in Cf specificity

Based on studies of RIs, solvent-exposed amino acid residues of the β-sheets present in LRR proteins are expected to form a recognition surface at the inner site of the curved

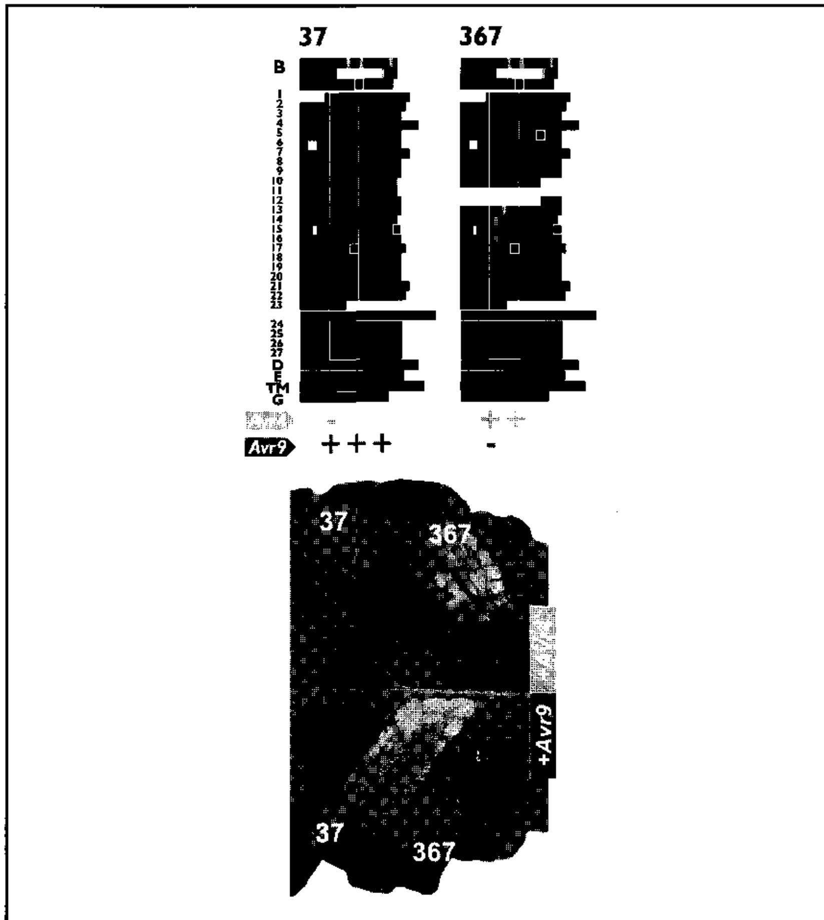


Figure 4. A Cf-9 mutant carrying the specificity determinants of Cf-4 confers recognition of AVR4. The specificity of mutant 37, which confers full AVR9 recognition upon transient expression in tobacco (Figure 2A), was changed into specificity for AVR4 by deleting LRRs 11 and 12 and introducing W389, G411, and F457, resulting in mutant 367. For further details, see legends to Figures 1 and 2.

LRR domain (Kobe and Deisenhofer, 1995). Thus, variation in the decoration of the recognition surface by substitution of solvent-exposed amino acid residues is likely to affect the recognitional specificity of an LRR protein. Consistent with this theory, it was found that Cf proteins and many R proteins of the NBS-LRR class are hypervariable at solvent-exposed positions in the LRR domain (Parniske et al., 1997; Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998; Noël et al., 1999; Bittner-Eddy et al., 2000). However, to date, the exact role of solvent-exposed residues in the specificity of R proteins has not been documented. We have now identified three solvent-exposed residues that are essential for full Cf-4 function. Two of these (W389 and G411) reside among the seven positions that have been reported to be hypervariable in Cf proteins

encoded by the *Cf-4* and *Cf-9* gene clusters (Parniske et al., 1997). Both W389 and G411 are unique among homologs of Cf-4 and are clustered in the Cf-4 protein. This finding indicates that diversifying selection of solvent-exposed amino acid residues was more important in the generation of AVR4 recognitional specificity than sequence exchange between Cf-4 progenitor genes.

We also showed that amino acid residues W389, G411, and F457 only partially contribute to Cf-4 specificity. Single W389Y and F457L substitutions reduced Cf-4 function only slightly, whereas Cf-4 function was abolished completely in our assays when W389 and F457 were substituted simultaneously. The slightly reduced activity of mutant 367, which contains the Cf-4–specific features that we have identified, might be explained by the absence of a combination of certain Cf-4–specific residues in the LRRs. When considered individually, these residues contribute only slightly to Cf-4 function, and therefore their relevance was not identified by analysis of mutants 129, 35, and 332 (Figures 2E and 3B). Possibly T433, which is present at a putative solvent-exposed position in LRR 15 of Cf-4, has such a slight contribution to Cf-4 function (Figure 3B, compare curves a and e). We also showed that double mutants can be very useful to identify amino acid residues that contribute only partially to Cf-4 function. In this way, the importance of amino acid residue W389 for Cf-4 function was easily revealed by gain-of-function studies, starting from inactive double mutants.

The role of solvent-exposed amino acid residues in LRR proteins also was addressed in reports on mutant LRR proteins that carry single amino acid residue substitutions at putative solvent-exposed positions. These proteins are encoded by alleles of *Cf-9* (D509N and S676L; Thomas et al., 1998), *clv1-4* and *clv1-8* (Clark et al., 1997), *rpp8-2* (McDowell et al., 1998), *rps5-1* (Warren et al., 1998), *rps2-201* (Bent et al., 1994; Mindrinos et al., 1994), and *rpm1-4* (Grant et al., 1995). All of these mutants appeared inactive or showed a severely reduced functionality. None of the observed substitutions was present in functional homologs. In the case of Cf-9 and RPS5, the substitutions were found in regions that are conserved among family members. Although these mutations at putative solvent-exposed positions may have a direct effect on interactions with other proteins, the phenotype also can be a result of effects of the mutation on overall protein structure or stability. Only in case of the RPS5 mutation, a role for the mutated amino acid residue can be speculated because this *rps5-1* mutation also partially suppresses the function of other homologous *R* genes in Arabidopsis (Warren et al., 1998).

The putative solvent-exposed positions of W389, G411, and F457 in LRRs of Cf-4 suggest that these residues play an important role at the surface where Cf-4 interacts with a protein or proteins of the AVR4 perception complex. The weak effect of the W389Y and F457L substitutions is possibly due to the high similarity between the side chain of the resident and the introduced amino acid residue. Amino acid substitutions also can affect the orientation of side chains of adjacent solvent-exposed amino acid residues, as was suggested for the V118G substitution in polygalacturonase-inhibiting protein 2 (Leckie et al., 1999). This finding may explain the effect of the G411E substitution, because glycine has no side chain that can participate in interactions.

The role of variation in R proteins

We have shown that of the 57 amino acid residues that differ between the LRRs of Cf-4 and Cf-9, 54 are not essential for Cf-4 specificity. This could be a general feature of LRR proteins. For example, a *Cf* gene mediating AVR9 recognition in *Lycopersicon pimpinellifolium* encodes a homolog that differs from Cf-9 by 63 amino acid residues (chapter 4). Again, most of the variation between Cf-9 and this functional Cf-9 homolog resides in the N-terminal half of the proteins, particularly at putative solvent-exposed positions in the β-sheets of the LRRs.

These observations raise the question of why R proteins carry so many variant amino acid residues in the LRR domain but only a few residues are required for recognitional specificity. One possibility is that the high number of variant residues at solvent-exposed positions is allowed because diversification changes only the decoration of the protein and not the overall structure. However, the reported diversifying selection on solvent-exposed residues suggests that there must be an additional advantage. We propose four distinct roles for variation in R proteins. First, variation in LRR proteins could be a result of selection in the past. This implies that the existing variation has no current function but remains as a relic from the evolution of new *R* gene specificities. Second, ongoing variation in LRRs of R proteins is still vital because it provides the basis for the generation of new specificities through recombination and gene conversion. Third, variation in LRRs of R proteins also might give plant populations the ability to recognise a diverse collection of non-self proteins. Finally, the versatile binding capabilities of LRR proteins also suggest that variable amino acid residues can be involved in recognition of multiple ligands, thus generating dual or multiple recognitional specificities on a single R protein.

Thus, R proteins could act as scaffolds that can easily change their decoration as a result of gene shuffling and diversifying selection on solvent-exposed amino acid residues. We have shown that most of the specific decorations found on an R protein are not necessarily involved in the determination of recognitional specificity of that protein. This finding indicates that the decoration of R proteins is not fully adapted to a certain function but rather reflects the fact that R proteins have the versatility to adapt to sense the presence of new "foreign" proteins. For plants, such a flexible recognition system for diverse pathogens is crucial for survival among adapting pathogens. The elucidation of where specificity resides in Cf proteins eventually might allow the design of custom R proteins to provide durable resistance by targeting recognitional specificity to conserved proteins of pathogens that are crucial for their pathogenicity.

Materials and Methods

DNA manipulations and plasmids

All DNA manipulations were performed using standard protocols (Sambrook et al., 1989). Polymerase chain reaction (PCR) was performed with *Pfu* polymerase (Stratagene, La Jolla, CA) or AmpliTaq polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Restriction enzymes, Klenow polymerase, T4 ligase, and *Escherichia coli* DH5α cells were from Life Technologies (Breda, The Netherlands). Primers were synthesised by Amersham-Pharmacia (Buckinghamshire, UK). The authenticity of all cloned PCR fragments was confirmed by sequencing. The presence of correct *Cf-9* and *Cf-4* fragments in binary constructs was assayed using PCR on plasmid DNA isolated from *Agrobacterium tumefaciens*, followed by restriction analysis of the amplified fragments to reveal polymorphic sites.

The following plasmids that were used in this study have been described previously (chapter 2): pRH46 (35S-driven *Cf-4*), pRH1 (35S-driven *Cf-9*), pRH48 (binary *Cf-4* vector), pRH21 (binary *Cf-9* vector), pRH87 (binary *Avr4* vector), pMOG978 (binary *Avr9* vector), and pMOG800 (binary vector).

Construction of binary plasmids

To construct a binary plasmid carrying 35S-driven *Cf* genes lacking the part encoding leucine-rich repeats (LRRs) 1 to 17, we removed *Bam*HI and *Cla*I sites from the multiple cloning sites of pRH1, resulting in pRH17. *Cf-4* and *Cf-9* fragments that lacked the part encoding LRRs 1 to 17 were amplified from pRH46 and pRH1, respectively. This was done with PCR overlap extension by using primers that anneal in the 35S promoter (5'-gttcatttcatttggagagg-3') and at the conserved *Hin*dIII site, which is present in the open reading frame at a position corresponding to LRR 21 of the encoded Cf protein (5'-catgcaacttatttgatctc-aagc-3') and overlap primers 5'-aacaatatcaataggcct-gtcgtctcgtcacaatcgatgccatcc-3' and 5'-gacgacaggcc-tattgatattgttagacttgggatccaataatttgg-3' (the *Cla*I and *Bam*HI sites, respectively, are underlined). The *Xba*I and *Eco*RI sites in the *Cf-4* sequence encoding the B-domain were removed using PCR overlap extension with overlap primers 5'-gctcttcttga-gttcaagaac-3' and 5'-gttcttgaactcaagaagagc-3' in such a way that the encoded protein sequence remained the same. The amplified *Cf* fragments lacking the part encoding LRRs 1 to 17 were cloned into pRH17 by using *Nco*I and *Hin*dIII restriction sites, resulting in pRH26 and pRH18 for *Cf-4* and *Cf-9*, respectively. The promoter–open reading frame–terminator cassettes of pRH26 and pRH18 were transferred subsequently to pMOG800 by using *Xba*I and *Eco*RI restriction sites, creating binary vectors pRH94 and pRH22, respectively. Mutants 353 and 352, encoding Cf-4 mutants with exchanges within the B-domain, were generated by PCR overlap extension by using overlap primers 5'-gcttctga-ttattgttacgac-3' and 5'-gtcgtaacaataatcagaagc-3'.

Mutagenesis in the LRR Domain

Mutations within the LRR domain were generated as follows. A construct encoding the LRRs of Cf-9, with the Cf-4-specific deletion of LRRs 11 and 12, was made by removing a 138 bp *Ava*II fragment encoding LRRs 11 and 12 from pRH1, resulting in pRH5. DNA fragments encoding LRRs 1 to 17 were amplified from pRH46, pRH1, and pRH5 by using primers that are described in Table 1. For some fragments, PCR overlap extension was used or cloned PCR products were used as a template. In a few *Cf-9* constructs, the *Eco*RI site present in the DNA encoding LRR 15 was removed by PCR overlap extension by using overlap primers 5'-gctgaaaggtcgtattccgaatttactcctaaaccagaagaacc-3' and 5'-ggttcttctggtttaggagtgaatttggaatacgacctttcagc-3' without changing the coding sequence. The fragments were cloned subsequently into pBluescript SK2 (Stratagene) by using *Cla*I, *Bam*HI, *Eco*RI, and *Xho*I restriction sites. LRR-encoding fragments were cloned subsequently into binary vectors pRH22 and pRH94, encoding Cf-9 and Cf-4 that lack LRRs 1 to 17. For some cloning steps, removal of *Xho*I and *Eco*RI restriction sites from the multiple cloning sites of the vectors pRH22 and pRH94 was required.

Agroinfiltration

Tobacco plants (*Nicotiana tabacum* cv. Petite Havana SR1) were grown under normal greenhouse conditions. Binary plasmids were transferred to *Agrobacterium tumefaciens* strain MOG101 (Hood et al., 1993) by electroporation. Culture preparation and infiltration of leaves of 4- to 8-week-old tobacco plants were performed as described previously (chapter 2).

For quantitative comparisons, Agrobacterium cultures that carry a plasmid that encodes a (mutant) Cf protein were mixed in different ratios with a culture of equal density carrying an AVR-encoding plasmid and infiltrated into opposite tobacco leaf halves. At 7 days after infiltration, outlines of sectors and necrotic areas were drawn on a sheet. Areas on the scanned sheet were quantified subsequently using the magnetic lasso and histogram options of Adobe Photoshop (version 5.0; Adobe Systems, Mountain View, CA). Each pair of curves represents one leaf. Differences in Cf-4 curves are due to difference in responsiveness of the infiltrated leaves. The dose-response curves that are shown are representative of at least four independent experiments.

Acknowledgments

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Table 1. Primers used to generate fragments encoding LRRs

Primer	Sequence (5′ to 3′)	*Cf-4*	*Cf-9*
AF (f)	ccaaaacattaagtgccgttactctaaaac	1123	
AR (r)	gttttagagtaacggcacttaatgttttgg	1142	
BR1 (r)	attattggatccccaagtctaacaatatc	1314	1485
CF1 (f)	ggcatcgattgtgacgagacg	215	245
CY (r)	acgtggatccgaattcgctcgagaggtaaagacattgtaggtt		1188
DF1 (f)	ataaccatcttgaaggaccaatt	933	966
DF2 (f)	ggbccaattccatccaac	947	1118
DR1 (r)	aattggtccttcaagatggttat	955	988
DR2 (r)	gttggatggaattggwcc	964	1135
EC (r)	acgtggatccgaattcgctcgagagccaaagacattctaggtt		1188
EF (f)	ccatcactgatagagttagacttgagc	1049	1220
ER (r)	gctcaagtctaactctatycagtgatggaaggg	1075	1246
FY (r)	acgtggatccgaattcgctcgagaggtaaagtaaaactaggtt		1188
GF (f)	ccatcactggttgggttagacttgagc	1049	1220
GR (r)	gctcaagtctaacccaaccagtgatggaaggg	1075	1246
QF4 (f)	acttctcgagtaacaacttgaatggg	995	
QF9 (f)	acttctcgagtaaccacttgaatggg	995	1166
QR4 (r)	ttaaggatcctctcgagagccaaagtatttgtagg	1018	
QR9 (r)	tcaaggatcctctcgagaggtagagacattctagg		1189
RR (r)	gagtgaattcggaatacgaccttttagc	1177	
SF1 (f)	gccgatcgataacatctcgtcgactattcctt	568	601
SR2 (r)	ccccggatccaggaatagtcgacgagatgtt	604	641

Forward (f) or reverse (r) primers are indicated, and restriction sites are underlined. The position of the 5′ end of the primer in *Cf-4* or *Cf-9* is indicated relative to the start codon of the open reading frame. Only primer positions that were used are indicated.

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Chapter 4

Intragenic Recombination Generated two Distinct *Cf* Genes that Mediate AVR9 Recognition in the Natural Population of *Lycopersicon pimpinellifolium*

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Abstract

Resistance gene *Cf-9* of cultivated tomato (*Lycopersicon esculentum*) confers recognition of the AVR9 elicitor protein of the fungal pathogen *Cladosporium fulvum*. The *Cf-9* locus, containing *Cf-9* and four homologs (*Hcr9*s), originates from *L. pimpinellifolium* (*Lp*). We examined naturally occurring polymorphism in *Hcr9*s that confer AVR9 recognition in the *Lp* population. AVR9 recognition occurs frequently throughout this population. In addition to *Cf-9*, we discovered a second gene in *Lp*, designated *9DC*, which also confers AVR9 recognition. Compared to *Cf-9*, *9DC* is more polymorphic, occurs more frequently and is more widely spread throughout the *Lp* population, suggesting that *9DC* is older than *Cf-9*. The sequences of *Cf-9* and *9DC* suggest that *Cf-9* evolved from *9DC* by intragenic recombination between *9DC* and another *Hcr9*. The fact that the 9DC and Cf-9 proteins differ in 61 amino acid residues, and both mediate recognition of AVR9, shows that in nature Hcr9 proteins with the same recognitional specificity can vary significantly.

Introduction

Recognition of a diverse range of pathogens, followed by an adequate defence response, is crucial for survival of plants. Resistance (*R*) genes, which mediate recognition of products of matching avirulence (*Avr*) genes, play a key role in recognition of pathogens (Flor, 1946). Most *R* gene products contain a leucine-rich repeat (LRR) domain, with putative solvent-exposed amino acid residues that decorate the surface of the protein where specific interactions with other proteins are thought to occur (Jones and Jones, 1997). R proteins with different specificity differ predominantly at putative solvent-exposed positions, which are often thought to result from adaptive evolution (Richter and Ronald, 2000).

Plants need to generate *R* genes with new specificities because pathogens continuously try to circumvent recognition by the host plant. New *R* genes are thought to evolve by sequence exchange between homologous genes and by accumulation of random point mutations in codons that encode amino acids located at putative solvent-exposed positions (Richter and Ronald, 2000; Michelmore and Meyers, 1998).

The continuous generation of new recognitional specificities by the host, followed by subsequent adaptation of the pathogen to circumvent this recognition, can be seen as an 'arms-race' between plants and pathogens (Dawkins and Krebs, 1979). Recent observations suggest that in nature, this 'arms-race' is a slow process and that the battle

between plants and pathogens is more likely similar to a 'trench-warfare'. In this model, frequencies of *R* genes in the plant population fluctuate in time, following the frequency of the matching *Avr* gene in the pathogen population (Stahl et al., 1999). Consistent with this model, gene-for-gene pairs like *AvrRpm1-RPM1* and *AvrPto-Pto* are ancient (Stahl et al., 1999; Reily and Martin, 2001) and plants carrying or lacking the *RPM1* gene co-exist in the plant population (Stahl et al., 1999).

The tomato resistance genes *Cf-9* and *Cf-4* mediate recognition of strains of the leaf mould fungus *Cladosporium fulvum* carrying the *Avr9* or *Avr4* gene, respectively (Joosten and De Wit, 1999). Recognition by resistant plants results in the activation of multiple defence responses that limit further fungal growth. The hypersensitive response (HR) is a macroscopically visible phenomenon, where plant cells surrounding the infection site quickly die. The *Avr9* and *Avr4* genes both encode proteins that are secreted by the fungus into the extracellular space of tomato leaves during infection of susceptible plants. Injection of these elicitor proteins into the extracellular space of tomato leaves carrying the matching *Cf* gene is sufficient to trigger HR. The *Cf* genes encode receptor-like proteins with extracellular LRRs and are predicted to be anchored in the plasma membrane (Jones and Jones, 1997). Cf-4 differs from Cf-9 in 67 amino acid residues and contains three deletions when compared with Cf-9 (Thomas et al., 1997). By exchanging domains between Cf-4 and Cf-9, we previously showed that specificity in Cf-4 resides in the N-terminal domain, the number of LRRs and three Cf-4-specific amino acid residues at putative solvent-exposed positions (chapter 3). In Cf-9, specificity is likely scattered throughout the LRR domain (chapter 3; Wulff et al., 2001).

The *Cf-9* gene is the first described member of a gene family, called *Hcr9*s (Homologs of *Cladosporium fulvum* resistance gene *Cf-9*), present on the short arm of chromosome 1 of tomato. Thus far, 18 *Hcr9*s have been reported (Parniske et al., 1997; Parniske and Jones, 1999). The *Cf-9* gene is the third *Hcr9* (*Hcr9-9C*) of a cluster of five homologs, named *Hcr9-9A* to *-9E*. The *Cf-9* locus has been introgressed into cultivated tomato (*Lycopersicon esculentum*) from its wild relative *L. pimpinellifolium* (*Lp*) (Tichelaar, 1984). *Lp* contains many different recognitional specificities for proteins of *C. fulvum* and was used as a rich germplasm for *Cf* resistance genes (Laugé et al., 2000). The natural habitat of *Lp* is a narrow, 2500 kilometres long coastal area of Ecuador and Peru, bordered by the Pacific Ocean and the Andes mountains (Warnock et al., 1991). *Lp* is predominantly self-fertilizing and previous studies on the genetic variation in this species showed that allele frequencies can differ significantly between regions of the *Lp* habitat (Rick et al., 1977).

The large genetic variation in the *Lp* population prompted us to investigate whether this population contains *Hcr9*s that are polymorphic, but still mediate recognition of the same elicitor protein of *C. fulvum*. If this is the case, we might get insight on how existing recognitional specificities are maintained in nature and how new specificities evolve. Here, we show that AVR9 recognition occurs frequently throughout the *Lp* population, suggesting that this trait did not evolve recently. In addition to *Cf-9*, we discovered a second gene, designated *9DC*, which also mediates AVR9 recognition. *Cf-9* likely evolved by intragenic recombination between *9DC* and another *Hcr9*. It appears that in nature, Hcr9 proteins that have the same recognitional specificity can be highly polymorphic.

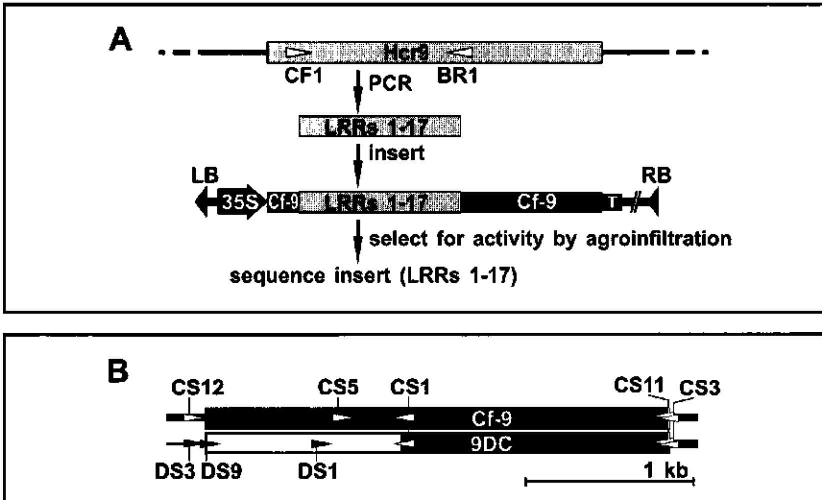


Figure 1. Selection procedure for chimeric *Hcr9* constructs that confer AVR9 recognition and position of the various specific primers.

A CF1 and BR1 are *Hcr9*-specific primers (triangles) located at positions corresponding to the B-domain and LRR 17, respectively, of the encoded Hcr9 protein. Amplified fragments encoding LRRs 1-17 were inserted into a binary expression vector that contains the *Cf-9* ORF lacking the fragment encoding LRRs 1-17. 35S, CaMV 35S constitutive promoter; T, nos terminator; RB and LB, right and left border of T-DNA, respectively. For further details see materials and methods.

B Position of specific primers in and around *Cf-9* and *9DC* ORFs. Triangles indicate annealing position and direction of primers relative to the ORF of *Cf-9* and *9DC*. For further details see materials and methods.

Results

Identification of a novel *Hcr9* that mediates AVR9 recognition in *Lp*

In a previous study, six accessions of *Lp* were identified as AVR9-responsive (Laugé et al., 2000). We chose one AVR9-responsive plant of accession LA1301 to characterize the *Hcr9* mediating AVR9 recognition. *Hcr9*s are highly similar and the encoded proteins predominantly differ in LRRs 1-17 (Parniske et al., 1997). We previously found that this region determines specificity in Cf-9 (chapter 3). To identify the *Hcr9* that confers AVR9 recognition in this *Lp* accession, a library of chimeric *Cf-9* genes was generated in a binary vector, with fragments encoding LRRs 1-17 amplified from genomic DNA of the AVR9-responsive plant (Figure 1A, see materials and methods). Transient co-expression of the chimeric *Cf-9* genes with *Avr9* in tobacco by agroinfiltration (chapter 2) was used to select for fragments that complemented *Cf-9* function. Of the 13 chimeric constructs tested, three conferred AVR9 recognition (data not shown). The DNA sequence of the inserts encoding LRRs 1-17 revealed that the 3'-part (0.4kb, encoding LRRs 12-17) was identical to *Cf-9* (*Hcr9-9C*), whereas the 5'-part (0.8kb, encoding LRRs 1-11) was nearly identical to *Hcr9-9D*, which is located directly downstream of *Cf-9* at the *Cf-9* locus (Figure 2). Therefore, the newly discovered gene was designated *9DC*.

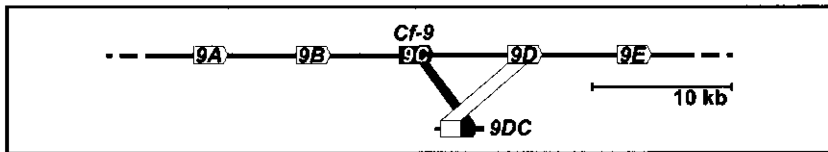


Figure 2. Organization of the *Hcr9*s at the *Cf-9* locus and their homology with *9DC*.
The five *Hcr9*s (*9A* to *9E*) present at the *Cf-9* locus are indicated (bottom) as well as the area of homology of *Hcr9-9C* and *Hcr9-9D* with *9DC* (top). Arrows indicate ORFs with transcriptional direction. Note that no mechanism nor direction in time is implied.

Based on sequence information of the fragment encoding LRR1-17, we expected the 5'-untranslated region (UTR) of *9DC* to be identical to *Hcr9-9D*, and the 3'-UTR to be identical to *Cf-9*. Using an *Hcr9-9D*-specific primer in the 5'-UTR and a *Cf-9*-specific primer in the 3'-UTR (primers DS3 and CS3, respectively, Figure 1B), we could indeed amplify the entire *9DC* ORF from genomic DNA of the AVR9-responsive plant. To test the encoded 9DC protein for mediating AVR9 recognition, the *9DC* ORF was inserted into a binary expression vector and co-expressed with *Avr9*. This demonstrated that *9DC* indeed confers AVR9 recognition (Figure 3A), whereas dilution experiments showed that its activity with respect to AVR9 recognition is similar to that of *Cf-9* (Figure 3B). Furthermore, injection of *9DC*-agroinfiltrated leaves with mutant AVR9 peptides that are either inactive (F21A), less active (F10A), or more active (R08K), as compared to wild-type AVR9 (Kooman-Gersmann et al.,1998), demonstrated that *9DC* confers AVR9 recognition with the same specificity as *Cf-9* (data not shown). Thus, *9DC* functions similarly to *Cf-9* in these assays.

The 5'-half of the *9DC* ORF (1966 bp) and 104 bp of the 5'-UTR, differ in only one nucleotide from *Hcr9-9D* (site x, Figure 4A), resulting in a difference in amino acid sequence as compared to the Hcr9-9D protein (site x, Figure 4B). The DNA-sequence of the 3'-half of the *9DC* ORF (1550 bp) and 26 bp of the 3'-UTR, also differs from *Cf-9* in only one nucleotide (site y, Figure 4A), which does not result in a difference in amino acid sequence. The 5'-end of the 31 bp recombination region (Figure 4A, bottom) is bordered by a *Cf-9*-specific nucleotide (t), whereas the 3'-end is bordered by an *Hcr9-9D*-specific insertion of three codons.

Most strikingly, the 9DC protein encoded by the *9DC* gene of *Lp* accession LA1301 differs in 61 amino acid residues from Cf-9 (Figure 4B). Of these residues, 45 are located in the first eleven LRRs, of which 22 are present at putative solvent-exposed positions. The 9DC protein also lacks three potential glycosylation sites. The amino acids that are polymorphic between 9DC and Cf-9 are similar in extent and position as those observed between Cf-4 and Cf-9 (Thomas et al., 1997). Nineteen amino acid residues of 9DC that differ from Cf-9 are identical to those occurring in Cf-4, of which five are located at putative solvent-exposed positions.

Molecular basis of AVR9 recognition in the *Lp* population

Having identified the gene that mediates AVR9 recognition in accession LA1301, we set out to examine the *Hcr9* mediating AVR9 recognition in other accessions of *Lp*. We took advantage of the collection of *Lp* accessions maintained at the University of California, Davis. Multiple plants of 231 accessions were injected with the AVR9 elicitor. Of 570

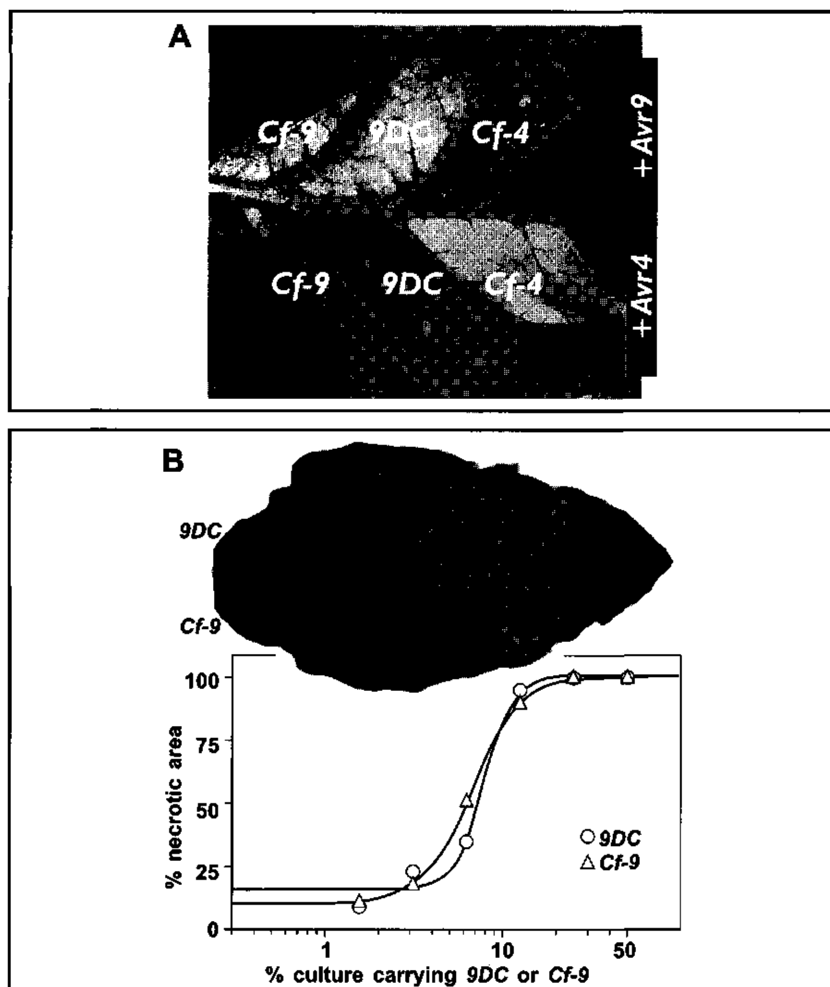


Figure 3. Comparative transient expression studies of *9DC* and *Cf-9*.

A *9DC* confers AVR9 recognition. An *Agrobacterium* culture carrying the *9DC* ORF in a binary expression vector was mixed with *Agrobacterium* carrying *Avr9* and infiltrated into a tobacco leaf sector. As controls, the ORFs of *Cf-9* and *Cf-4* were included and *Avr4* was used for co-expression. Photograph was taken at 7 days after infiltration.

B Activity of *9DC* and *Cf-9* in AVR9 recognition. *Agrobacterium* cultures carrying *9DC* or *Cf-9* were diluted in a culture carrying *Avr9* and infiltrated into neighbouring tobacco leaf sectors. Photograph was taken at 7 days after infiltration. The percentage of the infiltrated area that had become necrotic was measured and plotted against the percentage of culture containing *9DC* or *Cf-9*. Note that the dose-response curves for *9DC* and *Cf-9* are similar.

injected plants, 143 developed a specific HR. Responsive plants belong to 72 accessions, of which 27 contained both responsive and non-responsive plants. It appeared that AVR9-recognizing plants are present throughout the geographical distribution range of *Lp* (data not shown). To calculate frequencies of AVR9 recognition, the distribution range of

Lp was divided arbitrarily into four regions (regions A-D, Figure 5A). Interestingly, this revealed that the frequency of AVR9 recognition gradually increases in southern direction, up to an almost 3-fold higher level in the south when compared to the north of the *Lp* distribution range (Figure 5B).

From each accession containing AVR9-responsive plants, one responsive plant was randomly selected for genomic DNA isolation and subsequent PCR analysis. To detect *Cf-9* or *9DC*, primers were developed to specifically amplify fragments from *Cf-9* or *9DC*, but not from any other known *Hcr9* (lanes 0-5, Figure 5C). The identity of the amplified fragments was confirmed by sequencing. All AVR9-responsive plants contained either *Cf-9* or *9DC*, indicating that these are the only two genes that confer AVR9 recognition in the *Lp* population. None of the AVR9-responsive plants tested contained both *Cf-9* and *9DC*. Significantly, in accessions with both responsive and non-responsive plants, the *Cf-9* or *9DC* fragments were only detected in AVR9-responsive plants (Figure 5C). A *9DC* fragment was amplified from 56 of the AVR9-responsive plants, whereas from the remaining 16 a *Cf-9* fragment was amplified. Thus, *9DC* occurs more frequently in the *Lp* population than *Cf-9*. Accessions with *9DC* are present throughout the entire distribution range of *Lp*, whereas *Cf-9* is only found in accessions of *Lp* collected from northern and central Peru (Figure 5D).

Sequence polymorphism in *Cf-9* and *9DC*

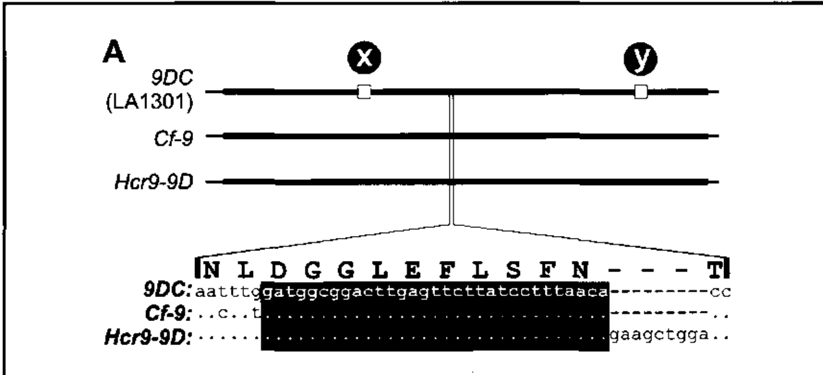
To determine whether polymorphism occurs in *Cf-9* and *9DC*, we cloned and sequenced six *Cf-9* ORFs and six additional *9DC* ORFs of different accessions, representing separated geographical collection sites (Figure 5A). All six *Cf-9* sequences were identical to the *Cf-9* ORF that was introgressed into *L. esculentum* (12). In contrast, DNA sequences of the six additional *9DC* ORFs showed three single nucleotide polymorphisms (SNPs) when compared to *9DC* of accession LA1301 (Figure 6). Two of these (sites 1 and 2) result in a polymorphic amino acid sequence (Figure 4B).

The SNPs differentiate the *9DC* genes into five different allelic classes (I-V, Figure 6). Alleles I and III were each identified in two separate accessions. Alleles I-IV may have

Figure 4. Comparison of nucleotide and protein sequences of *9DC*, *Cf-9* and *Hcr9-9D*. ►

A Schematic representation of the DNA sequences of *9DC* present in *Lp* accession LA1301, and *Cf-9* and *Hcr9-9D*. Thick lines indicate ORFs. Squares at positions x and y indicate nucleotides (C755T and T2160A, respectively) that are different from the DNA sequence of *Hcr9-9D* and *Cf-9*, respectively. The sequence with the recombination region (boxed in black) is enlarged. Dots (.) indicate nucleotides that are identical to *9DC*, minus (-) indicates nucleotides that are lacking. The amino acid sequence of the 9DC protein encoded by the area of recombination is indicated.

B Alignment of 9DC and Cf-9 proteins. Amino acid residues of 9DC and Cf-9 that are identical are shown in the central line. 9DC- and Cf-9-specific residues are shown in bold at top and bottom line, respectively. Potential N-glycosylation sites (NXS/T) in 9DC and Cf-9 are overlined and underlined, respectively. The amino acid sequence encoded by the recombination region (Figure 4A) is shown in italics. The box indicates the various ß-sheets (consensus XXLXLXX), each of which contains five putative solvent-exposed amino acid residues (X). The amino acid residue in the black box (site x) indicates the difference (Met-Thr) between the 9DC protein of LA1301 and the N-terminal half of the Hcr9-9D protein. Residues in white boxes are polymorphic in different *9DC* alleles: site 1 (Val-Ile) and site 2 (Ser-Phe), see Figure 6. Amino acid residues encoded at SNPs y and 3 (Figures 4A and 6) are not indicated since these do not result in a polymorphic amino acid sequence. SP, signal peptide; B, B-domain; 1-27, LRR-domain; D, D-domain; E, acidic domain; TM, transmembrane domain; G, cytoplasmic tail.



B

SP M[G/D]CVKLVF[F/L]MLY[V/T]FL[F/C]QL[VS/AL]SSS
B LPHLCPEDQAL[A/S]LLQFKNMFT[Y/L]N
PNA[FH/SD]YC[P/Y]DI[TG-RE/RTYVD]IQSYPRTL
SWNKSTSCCSWDGVHCDETTGQ ①
1 VIALDLRCSQLQGKFHSNSS
2 LFQLSNLKRLDLS[N/F]NNF[I/T]GSLISPK
3 FGEFS[D/N]LTHLDLS[D/H]SSFTG[V/L]IPSE
4 I[S/C]HLSKLHVL[L/R]I[G/C]DQYGLS[I/L]VP[H/Y]NFE[P/L]L
5 LKNLTQLRELNL[YE/ES]VN[L/I]SST[V/I]PS
6 NFSSHLTTLQLSGT[G/E]L[R/H]G[L/I]LPER
7 VFHLS[D/N]L[EF/QS]L[D/H]LS[Y/V]N[S/P]QL[M/T]VRFPTTK ②ⓧ
8 WNSSASLM[K/T]LYV[H/D]SVNIADRIP[E/K]S
9 FSHLTSLHEL[D/Y]MG[YT/RC]NLSGPIPKP
10 LWNLTNI[ES/VF]L[D/H]L[RY/GD]HLEGPI[PQ/SH]
11 [LP/FT]IFEKLK[K/R]LSL[FR/V-]N[D/N]N[L/F]*DGGLEFLSF*
12 *N*---TQLERLDLSSNSLTGPIPSN
13 ISGLQNLECLYLSSNHLNGSIPSW
14 IFSLPSLVELDLSNNTFSGKIQEF
15 KSKTLSAVTLKQNKLKGRIPNS
16 LLNQKNLQLLLLSHNNISGHISSA
17 ICNLKTLILLDLGSNNLEGTIPQCV
18 VERNEYLSHLDLSKNRLSGTINTT
19 FSVGNILRVISLHGNKLTGKVPRS
20 MINCKYLTLLDLGNNMLNDTFPNW
21 LGYLFQLKILSLRSNKLHGPIKSSGN
22 TNLFMGLQILDLSSNGFSGNLPERI
23 LGNLQTMKEID
ESTGFPEYISDPYDIYYNYLTTISTKGQDYDS
24 VRILDSNMIINLSKNRFEGHIPSI
25 IGDLVGLRTLNLSHNVLEGHIPAS
26 FQNLSVLESLDLSSNKISGEIPQQ
27 LASLTFLEVLNLSHNHLVGCIPKG
D KQFDSFGNTSYQGNDGLRGFPLSKLCGG
E EDQVTTPAELDQEEEEEDSPMISWQ
TM GVLVGYGCGLVIGLSVIYIMWSTQYPAWFS
G RMDLKLEHIITTKMKKHKKRY

evolved from each other by consecutive accumulation of point mutations (Figure 6). However, the combination of SNPs in allele V suggests that this allele has resulted from recombination between different *9DC* alleles. The geographical distribution of the *9DC* alleles does not reveal any direction of genetic drift (Figure 5A).

Discussion

Tremendous efforts in the past decade have resulted in the cloning of many *R* genes that confer recognition of very different pathogens. However, how *R* genes are generated and maintained in nature is still poorly understood. Most knowledge on evolution of *R* genes comes from comparison of *R* genes with different recognitional specificities. In this report, we examined naturally occurring polymorphism between *R* genes with the same recognitional specificity. The two genes that confer AVR9 recognition in the *Lp* population encode highly polymorphic proteins, but one likely evolved from the other by a single intragenic recombination event. Maintenance of both *Cf* genes in the *Lp* population is likely a result of 'trench-warfare', where the frequency of *Avr9* in the pathogen population is counterbalanced by the frequency of the matching *Cf* gene in the plant population.

Role of amino acid polymorphism in Cf proteins

Cf proteins differ predominantly in amino acid residues at putative solvent-exposed positions, which may be a result of adaptive evolution to mediate recognition of a particular avirulence factor (Richter and Ronald, 2000). However, we have previously shown that from the 67 amino acid residues that vary between Cf-4 and Cf-9, only three Cf-4-specific residues present at putative solvent-exposed positions are essential to confer AVR4 recognition (chapter 3). A comparison between Cf-9 and 9DC proteins described in this report reveals that significant variation in amino acid residues is also allowed for AVR9 recognition in

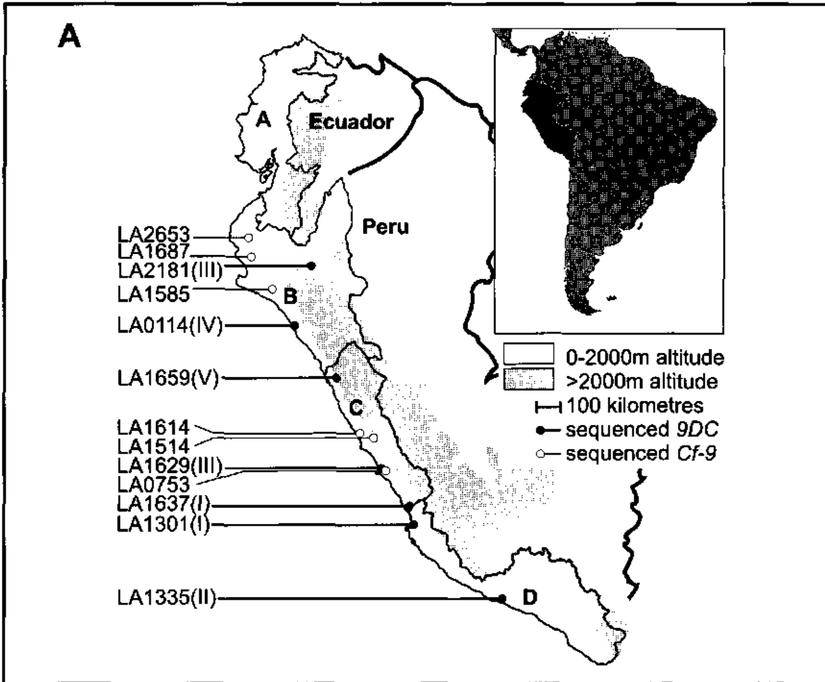
Figure 5. Frequency of AVR9-recognition and occurrence of *9DC* and *Cf-9* in the *Lp* population. ➤

A The natural distribution range of *Lp*. The *Lp* distribution range is bordered by the Pacific Ocean in the West, and the 2000 m elevation line of the Andes Mountains in the East. This area is divided into four regions: Ecuador (A) and northern (B), central (C) and southern (D) Peru. Accessions from which entire *9DC* or *Cf-9* ORFs have been sequenced are indicated on the left, with allelic classes I-V between brackets (see Figure 6).

B Frequency of AVR9-responsive plants per region. For each region, the number of AVR9-responsive plants was divided by the total number of AVR9-injected plants originating from that region. Not all plants could be mapped to regions.

C Amplification of fragments of *Cf-9* and *9DC*. Specific primers were tested (lanes 0-5) and used to detect the presence of *9DC* and *Cf-9* genes in *Lp* accessions that contain both AVR9-responsive and non-responsive plants (panels marked wit LA numbers). Specific amplification products of *Cf-9*, *9DC* and *Act* (control) genes were obtained as explained in materials and methods. Templates were genomic DNA isolated from: 1, MoneyMaker (MM)-Cf0 tomato; 2, MM-Cf9 tomato; 3, AVR9-responsive *Lp* plant of accession LA1301; 4, *Cf-9*-transgenic MM-Cf0 tomato; and 5, *Hcr9-9D*-transgenic MM-Cf0 tomato. Water (lane 0) was used as a negative control. AVR9-responsiveness is indicated with (-) or (+) below the panels.

D Frequency of *9DC* and *Cf-9* genes per region. One AVR9-responsive plant of each accession was analysed for the presence of *9DC* or *Cf-9* genes. The number of accessions that contained *9DC* or *Cf-9* was divided by the total number of accessions from that region. The frequency has been adjusted for the number of plants tested per accession. Not all accessions could be mapped to regions.


A
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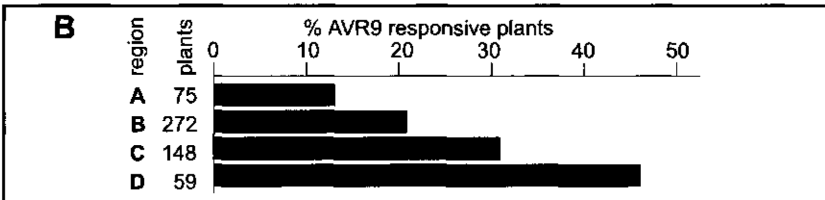
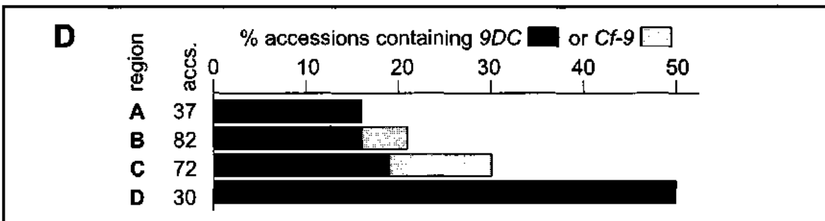
Ecuador
Peru
B
C
D
LA2653
LA1687
LA2181(III)
LA1585
LA0114(IV)
LA1659(V)
LA1614
LA1514
LA1629(III)
LA0753
LA1637(I)
LA1301(I)
LA1335(II)
0-2000m altitude
>2000m altitude
100 kilometres
sequenced 9DC
sequenced Cf-9


B
region
plants
% AVR9 responsive plants
0
10
20
30
40
50
A 75
B 272
C 148
D 59

C
0 1 2 3 4 5
LA1585
LA1687
LA2656
control
Cf-9
response: - + + + - - - + + - - - - + + - + + + - - + +
0 1 2 3 4 5
LA1590
LA1280
LA1629
control
9DC
response: - + + + - - + + - + + + - + + + - + - + - + -


D
region
accs.
% accessions containing 9DC or Cf-9
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A 37
B 82
C 72
D 30

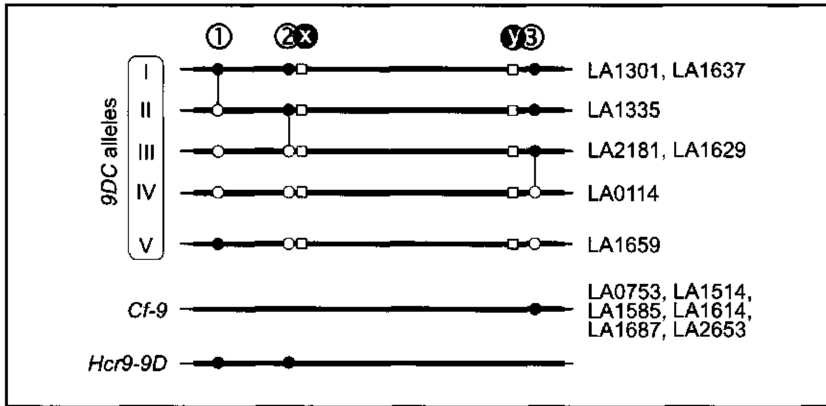

Figure 6. Polymorphism among *9DC* alleles and their relation with *Cf-9* and *Hcr9-9D*.
Accessions from which *9DC* or *Cf-9* have been sequenced are indicated on the right. Solid lines represent DNA sequences of the *9DC* alleles. Thick lines indicate ORFs. Open squares at positions x and y indicate the nucleotide that is conserved among *9DC* genes (see also Figure 4A). Circles at positions 1, 2 and 3 indicate SNPs (G244A, C713T and T2304C, respectively) between *9DC* ORFs, which differentiate these ORFs into five distinct allelic classes. Alleles I-IV are related to each other by accumulation of point mutations (vertical connections). Allele V likely resulted from recombination between two different *9DC* alleles. *Cf-9* contains a 3'-half that is identical to that of alleles I-III, except for site y. *Hcr9-9D* contains a 5'-half that is identical to that of allele I, except for site x.

nature. These results suggest that the variation observed between Cf proteins that mediate recognition of different avirulence factors is not a result of adaptive evolution. Variation may rather serve as a reservoir of diversity that facilitates the generation of R proteins with new specificities resulting from recombination and additional point mutations. These events can result in the sudden appearance (and disappearance) of a functional *R* gene according to the 'birth-and-death' model of evolution as postulated by Michelmore and Meyers (1998).

The origin of *Cf-9*

The sequences of both *9DC* and *Cf-9* are nearly identical in their 3'-halves, indicating that they are evolutionarily related by an intragenic recombination event. This also suggests that *9DC* maps at the same position as *Cf-9* in the tomato genome. Indeed, a testcross between accessions PI126915 (containing *Cf-9*) and PI126946 (containing *9DC*) indicated that *Cf-9* and *9DC* map at the same chromosomal position (Boukema, 1980; M. Kruijt, unpublished results). The observation that *9DC* occurs more frequent, is more dispersed in the *Lp* population and contains more sequence polymorphism when compared to *Cf-9*, suggests that the *9DC* gene is older than *Cf-9* and that *9DC* is an ancestor of *Cf-9*.

Thus, intragenic recombination between *9DC* and another *Hcr9* has likely resulted in *Cf-9*, which contains only the 3'-half of *9DC*, but still mediates AVR9 recognition. The 5'-half of *9DC* apparently ended up at the same locus as part of *Hcr9-9D*. *Hcr9-9D* does not confer AVR9 recognition (Figure 5C), probably due to absence of specific amino acids that are required for AVR9 recognition, as previously identified by Cf-4 and Cf-9 domain-swap analysis (chapter 3; Wulff et al., 2001).

Introgression of *Cf-9,* instead of *9DC,* into cultivated tomato has been a matter of chance. Accessions that contain *9DC,* like PI126946, have been used in breeding programs (Boukema, 1980; Laugé et al.,1998b). Indeed, one AVR9-responsive commercial tomato cultivar was found to contain *9DC* instead of *Cf-9* (R. Luderer, M. de Kock and M. Kruijt, unpublished results).

Intragenic recombination between *R* gene homologs

Intragenic recombination has been reported for many *R* gene families and is thought to be an important evolutionary force that generates new specificities. However, intragenic recombination resulting in new recognitional specificities has only been reported for *L* genes of flax (Ellis et al., 1999). Most intragenic recombination events described in literature were identified during screens for loss-of-function mutants of *R* genes. For example, intragenic recombination between *Cf-2* and *Hcr2-5B* resulted in a homolog that was not functional in AVR2 recognition (Dixon et al., 1998). Also, intragenic recombination between the functional *RPP8* gene and its adjacent homolog *RPP8A* probably resulted in an inactive *rpp8* homolog (McDowell et al., 1998). By searching for polymorphism in *Hcr9*s conferring AVR9-recognition in the *Lp* population, we have shown that intragenic recombination also occurs in nature, without having effect on recognitional specificity of the newly generated *Hcr9*.

AVR9 recognition in the *Lp* population

The high frequency of AVR9-responsive plants in the *Lp* population suggests that the locus mediating AVR9 recognition provides a selective advantage in nature. This has also been observed in modern resistance breeding where the *Cf-9* locus, which originates from *Lp*, has not yet been overcome by a fit, virulent isolate (Joosten and De Wit, 1999). The selective advantage of the *Cf-9* or *9DC* locus can be due to conferring AVR9 recognition itself, or can be the result of the presence of additional linked *R* genes with recognitional specificities for yet unidentified *Avr* gene products of *C. fulvum* (Parniske et al., 1997; Laugé et al., 1998a).

An interesting observation is that AVR9 recognition occurs almost 3-fold more frequent in the southern than in the northern regions of the *Lp* distribution range. Perhaps this reflects differences in pathogen pressure in these regions, which may be a result of climatic differences, favoring incidence of *C. fulvum*. Coastal temperatures in southern Peru range from 15-22 degrees compared to 18-25 degrees in Ecuador (Warnock, 1970). A more moderate temperature is known to favor the occurrence of tomato leaf mould (Small, 1930).

AVR9 recognition in the *Lp* population complies with 'trench-warfare' model

Previous studies on the presence of multiple genetic markers in the *Lp* population revealed that the largest genetic variation exists in northern Peru (Rick et al., 1977). In this area, *Lp* is a facultative outcrosser, which correlates with the presence of large flowers to attract bees, and long stamens that prevent self-pollination. In Ecuador and central and southern Peru, *Lp* is genetically more uniform and mainly self-fertilizing. These observations led to the hypothesis that northern Peru is the centre of origin of *Lp*, from which the species has spread in both northern and southern direction, giving self-fertilizing plants a selective advantage as pioneers. The study of Rick and co-workers (Rick et al., 1977) also revealed that certain alleles only occur in certain regions of the *Lp* distribution range.

In contrast, we have shown that AVR9 recognition occurs throughout the entire *Lp* distribution range. The predominantly self-fertilizing nature of *Lp* may be reflected in the accumulation of point mutations in *9DC* alleles I-IV. However, recombination between *9DC* alleles, resulting in allele V, has probably occurred in an outcrossing population. A previous study showed that unequal crossing-over at the *Cf-9* locus occurs more frequently in heterozygous plants than in homozygous plants (Parniske et al., 1997). This may suggest that intragenic recombination leading to *Cf-9* and *Hcr9-9D* occurred in a heterozygous background of an outcrossing population. Taken together, these observations suggest that AVR9 recognition was present in the center of origin of *Lp* before the species started to spread. This implies that AVR9 recognition is a trait that did not evolve recently. In addition, we observed that AVR9-recognising and non-recognizing plants co-exist in the same area (Figures 5B and C). These observations fully comply with the 'trench-warfare' model of gene-for-gene interactions between plants and pathogens (Stahl et al., 1999). According to this model, *R* genes are maintained in the plant population with a frequency that fluctuates in time, following the frequency of the matching *Avr* gene in the pathogen population. This model also implicates that *R* gene frequencies significantly differ between different areas. However, we observed a gradual decline in the frequency of AVR9 recognition in northern direction of the *Lp* distribution range. We believe that the regional *R* gene frequency is an average of fluctuating *R* gene frequencies of local populations. The *R* gene frequency at regional level may represent an equilibrium that does not fluctuate significantly in time. In either case, it is conceivable that 'trench-warfare' between plants and pathogens maintains *R* genes with a particular recognitional specificity in a natural plant population over a long period of time.

Materials and Methods

Accessions of *Lp* were donated by the C. M. Rick Tomato Genetic Resources Centre of the University of California (http://tgrc.ucdavis.edu/). Transgenic tomato plants (*L. esculentum* cv. MoneyMaker) carrying *Cf-9* or *Hcr9-9D* were a gift from Dr. J. Jones (Sainsbury Laboratory, Norwich, UK). Plants were grown under normal greenhouse conditions. To select for AVR9-responsive plants, leaflets were injected with 10µg/ml AVR9. HR was visible within two days after injection. The wild-type and mutant AVR9 proteins F21A, F10A and R08K used for injections have been described previously (Kooman-Gersmann et al., 1998).

DNA manipulations were performed using standard protocols (Sambrook et al., 1989). Polymerase chain reactions (PCRs) were performed with either *AmpliTaq* (Perkin-Elmer Applied Biosystems, Foster City, CA), *Pfu* (Strategene, La Jolla, CA) or with the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Restriction enzymes, T4 ligase, and *Escherichia coli* DH5α cells were from Life Technologies (Breda, the Netherlands). Primers were synthesized by Amersham-Pharmacia (Buckinghamshire, UK). Primer sequences are given in 5′ to 3′ direction, followed by the position of the 5′-end of the primer, relative to the ATG of *Cf-9*. Restriction sites are underlined. CF1: ggcatcgattgtgacgagacg, 245; BR1: attattggatcccaagtctaacaatatc, 1485; CS1: gccgttcagttgggtgtt, 1093; CS3: tctgaaagataatgatcaagtg, 2639; CS5: tttccaacttacaatcccttc, 713; CS11: cccccctgcagtcactaatatcttttcttgtgc, 2606; CS12: tcttctctatcaacataacaag, -44; DS1: gagagctcaacctttacgaa, 587; DS3: ctatgtgaggtagctagtag, -124; DS9: tttttccatgggttgtgtaaaacttgtg, -7. For construction of chimeric *Hcr9*s, genomic DNA was isolated (Van der Beek et al., 1992) from *Lp* plants and used as a template for PCR. Fragments of *Hcr9*s encoding LRRs 1-17 were amplified using primers CF1 and BR1, and cloned into pRH22 (chapter 3) using *Cla*I and *BamH*I restriction sites, thereby constructing chimeric *Hcr9*s (Figure 1A).

To detect *9DC* or *Cf-9* in *Lp* plants, genomic DNA was used as a template for PCR with specific primers (Figure 1B). Primers CS5 and CS1 were used to amplify a 378 bp *Cf-9*-specific fragment, whereas primers DS1 and CS1 were used to amplify a 507 bp *9DC*-specific fragment. In the same reaction mix, primers 5′-ggttatcttatggctactctg-3′ and

5'-gcgccatccgaatgtagag-3' were included to amplify a 778 bp fragment of the aspartate carbamoyl transferase (*Act*) gene that served as a positive control for the amplification reaction (Overduin et al., 1993).

The complete *9DC* and *Cf-9* open reading frames (ORFs) were amplified from genomic DNA by PCR using primers DS3 and CS3 or CS12 and CS3, respectively (Figure 1B). Amplified fragments were cloned into pGEM-T Easy (Promega, Madison, USA) and sequenced. The presence of polymorphic sites in the sequences was determined unambiguously by sequencing the PCR products directly or by sequencing independent clones.

To clone the *9DC* ORF into a binary expression vector, primers DS9 and CS11 were designed (Figure 1B). Amplified fragments were inserted between the 35S promoter and terminator (T) of pRH80 (chapter 2), using *Nco*I and *Pst*I restriction sites. The 35S-*9DC*-T cassette was subsequently inserted into the binary plasmid pMOG800 (chapter 2), using *Xba*I and *Kpn*I restriction sites.

Agroinfiltration of tobacco plants (*Nicotiana tabacum* cv. Petite Havana SR1) was performed as described (chapter 2). To compare the activity of *9DC* with *Cf-9*, dilution series of *Agrobacterium* cultures were infiltrated and necrotic responses were quantified as described (chapter 3).

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Chapter 5

The C-terminal Dilysine Motif for Targeting to the Endoplasmic Reticulum Is Not Required for Cf-9 Function

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Abstract

The tomato resistance gene *Cf-9* encodes a membrane-anchored, receptor-like protein that mediates specific recognition of the extracellular elicitor protein AVR9 of *Cladosporium fulvum*. The C-terminal dilysine motif (KKRY) of Cf-9 suggests that the protein resides in the endoplasmic reticulum. Previously, two conflicting reports on the subcellular location of Cf-9 were published. Here we show that the AARY mutant version of Cf-9 is still functional in mediating AVR9 recognition, suggesting that functional Cf-9 resides in the plasma membrane. The data presented here and in reports by others can be explained by masking the dilysine signal of Cf-9 with other proteins.

The interaction between tomato and the pathogenic biotrophic fungus *Cladosporium fulvum* is a well-established model in which to study molecular aspects of host resistance and pathogen avirulence (Joosten and De Wit, 1999). In this interaction, gene-for-gene-based recognition events result in a hypersensitive response (HR) and host resistance. Avirulence gene *Avr9* of *C. fulvum* encodes a race-specific elicitor that is secreted by the fungus during its growth in the extracellular space of the tomato leaf (Van Kan et al., 1991). *Cf-9*, the matching resistance gene of tomato, encodes a receptor-like protein with leucine-rich repeats (LRRs), a transmembrane domain, and a short C-terminal domain that lacks motifs for downstream signalling (Jones et al., 1994). Transformation of tobacco, potato, petunia, and several *Nicotiana* species with *Cf-9* showed that this gene is essential and sufficient to confer specific recognition of AVR9 (Hammond-Kosack et al., 1998; chapter 2).

Considering the key role of Cf-9 in mediating perception of the extracellular AVR9 protein, it is anticipated that Cf-9 resides in the plasma membrane with its LRRs protruding in the extracellular space. The presence of a C-terminal dilysine motif (KKRY), however, predicts a different location for the Cf-9 protein. As seen in yeast and mammals, this type of motif confers endoplasmic reticulum (ER) localisation of membrane-anchored proteins through retrieval and retention mechanisms (Andersson et al., 1999; Teasdale and Jackson, 1996). To elucidate the role of Cf-9 in AVR9 perception and subsequent activation of HR, it is crucial to know the exact location of the Cf-9 protein within the plant cell. Recently, two conflicting reports on the subcellular location of Cf-9 were published. One report shows that Cf-9 is localised in the plasma membrane (Piedras et al., 2000), whereas another demonstrates that the dilysine motif of Cf-9 is functional and the protein resides in the ER (Benghezal et al., 2000). The latter study also shows that the ER retrieval-retention mechanism is saturable, raising serious doubts over the conclusions drawn by Piedras et al. (2000), who used *Cf-9* transgenes that were driven by the strong CaMV 35S promoter.

Thus far, the requirement of the KKRY motif for the function of Cf-9 has not been examined. In this study, we examined the effect of mutations in the KKRY motif on Cf-9 function. Co-expression of *Cf-9* with *Avr9* by agroinfiltration of tobacco leaf sectors results in an HR that is visible as necrosis (chapter 2) and was used as an assay to test the function and activity of mutant Cf-9. To determine whether the dilysine motif is essential for Cf-9 function, we changed the KKRY sequence of Cf-9 into AARY (Figure 1A). Significantly, in the presence of AVR9, agroinfiltration of Cf-9-AARY showed that this mutant is still functional in AVR9 recognition (Figure 1B).

If Cf-9 is functional in the ER, then activity of the Cf-9-AARY mutant that lacks the ER retrieval–retention signal could be the result of overexpression, by which the secretion pathway is saturated and part of the Cf-9-AARY proteins remains in the ER. We therefore examined the activity of the Cf-9-AARY mutant at lower expression levels. During agroinfiltration, Agrobacterium cells are present in excess compared with plant cells, which results in the transformation of nearly every cell of the infiltrated tissue (Kapila et al., 1997). Dilution of the cultures likely results in a decrease in the number of T-DNA transfers to each cell, resulting in decreased expression levels. This quantitative assay was introduced previously (chapter 2) and was validated further by demonstrating that Cf-4 mutants carrying single amino acid substitutions showed a slightly reduced activity when compared with wild-type Cf-4 (chapter 3). Quantification of necrotic areas obtained from infiltration of dilution series showed that the Cf-9-AARY mutant is only slightly less active as wild-type Cf-9 (Cf-9-KKRY) at lower expression levels (Figure 1C). Therefore, the activity of the Cf-9-AARY mutant is not likely to result from overexpression.

These data indicate that the dilysine motif in Cf-9 is somehow ignored by the ER retrieval-retention mechanism. Studies on translocation of proteins in yeast showed that phenylalanine (F) or tyrosine (Y) residues at the C terminus can weaken the ER retrieval-

Figure 1. Functionality and activity of Cf-9 mutants. ➤

A Schematic representation of the Cf-9 protein encoded by open reading frames (ORFs) of the (mutant) Cf-9 constructs used in this study. Genes are present on the T-DNA of binary plasmids and ORFs encoding (mutant) Cf-9 are driven by the CaMV 35S promoter. The four C-terminal amino acid residues are shown. Two mutations in the KKRY motif are boxed. The C-terminal ISWQ sequence of the Cf-9-ΔTMG mutant represents the C-terminal end of the E domain (Jones and Jones, 1997). Wild-type Cf-9 (Cf-9-KKRY) is encoded by binary plasmid pRH27, which was generated by cloning a fragment encoding LRRs 1-17 of Cf-9 into binary plasmid pRH22 that contains *Cf-9* lacking the sequence encoding LRRs 1-17 with the *Cla*I and *Bam*HI restriction sites (chapter 3). Cf-9 mutants were generated by polymerase chain reaction with pRH27 as template and forward primer 5′-gcattattggagatcttgttgg-3′ (*Bgl*II site underlined) and reverse primers 5′-agctctgcagctaatatcttgccgcgtgctttttcattttcgtag-3′, 5′-agctctgcagctaagctgctttctt-gtgctttttcattttcgtag-3′, and 5′-aaaactgcagctactgccaactgatcattggtg-3′ (*Pst*I sites underlined), thereby generating mutants Cf-9-AARY, Cf-9-KKAA, and Cf-9-ΔTMG, respectively. Amplified fragments were cloned into pRH18, which encodes Cf-9 lacking LRRs 1-17 (chapter 3), with the *Bgl*II and *Pst*I restriction sites. Fragments with the correct sequence were cloned into pRH27 with the *Bam*HI and *Eco*RI restriction sites, resulting in pRH386, pRH377, and pRH380, respectively. These vectors and pRH27 were transferred to *Agrobacterium tumefaciens* (strain MOG101), which was used for agroinfiltration. SP, signal peptide; B, B domain; LRRs, domain containing 27 leucine-rich repeats; D, D domain; E, E domain; TM, transmembrane domain; G, short C-terminal domain.

B Transient expression of (mutant) Cf-9 proteins in tobacco. Agroinfiltration of transgenic cultures of Agrobacterium carrying Cf-4- and various (mutant) Cf-9-encoding constructs into

A

SP B LRRs D E TM G

Cf-9-KKRY -KKRY

Cf-9-AARY -AARY

Cf-9-KKAA -KKAA

Cf-9-ΔTMG -ISWQ

B

Cf-4, Cf-9-KKRY, Cf-9-AARY, Cf-9-KKAA, Cf-9-ΔTMG (+ Avr9)

Cf-4, Cf-9-KKRY, Cf-9-AARY, Cf-9-KKAA, Cf-9-ΔTMG (+ Avr4)

C

% necrotic area (0, 25, 50, 75, 100)

Cf-9-KKRY, Cf-9-AARY; Cf-9-KKRY, Cf-9-KKAA

% culture carrying (mutant) Cf-9 (1, 5, 10, 50)

Nicotiana tabacum cv. Petite Havana SR1 was performed as described (chapter 2). For transient co-expression of *Cf-4* and (mutant) *Cf-9* genes with *Avr9* or *Avr4*, Agrobacterium cultures containing each of the genes were mixed in a 1:1 ratio and infiltrated into a leaf sector of tobacco. Photograph was taken at 7 days post infiltration (dpi) and represents four independent assays.

C Quantitative comparison of the necrosis-inducing activity of wild-type Cf-9 (Cf-9-KKRY) with Cf-9-AARY (upper panel) and Cf-9-KKAA (lower panel) mutants. Agrobacterium cultures carrying the constructs were mixed in different ratios with a culture of equal density carrying an AVR9-encoding plasmid and infiltrated into sectors of opposite tobacco leaf halves (chapter 2). The percentage of the infiltrated area that had become necrotic at 7 dpi was measured and plotted against the concentration of Agrobacterium that carries the plasmid-encoding (mutant) Cf-9. Curve represents nine curves obtained from three independent triplicate experiments.

retention mechanism (Itin et al., 1995). The presence of a tyrosine in the KKRY motif may therefore reduce the effect of the dilysine motif. To study the role of the two C-terminal amino acid residues in Cf-9, we changed the KKRY motif into KKAA (Figure 1A). This Cf-9-KKAA mutant appeared to be fully functional (Figure 1B), and its activity is only slightly reduced when compared with wild-type Cf-9 (Cf-9-KKRY) (Figure 1C), demonstrating that weakening the effect of the dilysine motif by the C-terminal tyrosine is not compromising Cf-9 function.

Another reason why the Cf-9-AARY mutant is still functional could be the absence of the dilysine motif in the mature Cf-9 protein. LRR proteins that are not anchored in the membrane can still be functional in recognition. Examples are the resistance gene product Xa21D (Wang et al., 1998) and polygalacturonase inhibitor proteins (De Lorenzo and Cervone, 1997). Interestingly, heterologous expression of the Cf-9-NNRY mutant in tobacco BY-2 cells results in a similar truncated protein consisting of domains B to E (Figure 1A) that remains associated with the membrane fraction (Benghezal et al., 2000). In order to test whether Cf-9 protein lacking the transmembrane domain is still functional, we deleted the TM and G domains from Cf-9, resulting in mutant Cf-9-ΔTMG (Figure 1A). This mutant was no longer functional in AVR9 recognition (Figure 1B), indicating that the membrane anchor is required for Cf-9 function.

The most plausible explanation for the dilysine motif not to be essential for Cf-9 function is that other proteins mask this motif. A similar escape from the ER retrieval-retention machinery has been described for the human receptor complex for immunoglobulin E, in which the α-chain contains a dilysine motif that is sterically masked after association with the γ-chain on the ER membrane (Letourneur et al., 1995). This masking is thought to result in quality control at the level of the ER, discriminating between assembled and unassembled receptors. Only fully assembled receptor complexes in which the dilysine motif is masked can leave the ER. The dilysine motif of Cf-9 might function in a similar way, assuring that only Cf-9 protein present in a functional signalling complex integrates in the plasma membrane. Thus, non-functional Cf-9 protein remains in the ER, whereas functional Cf-9 protein present in a complex is transported to the plasma membrane. The slightly reduced activity of the Cf-9-AARY mutant might be a result of a quicker exit from the ER of this protein, thus interfering with the formation of functional complexes.

We suggest two candidate proteins that could mask the KKRY motif of Cf-9 in a complex. Because Cf-9 does not appear to bind directly to AVR9 (R. Luderer et al., 2001), it seems likely that perception of AVR9 requires the high-affinity binding site (HABS) for AVR9 that is present in plasma membranes of solanaceous plants (Kooman-Gersmann et al., 1996). This HABS possibly could mask the dilysine signal upon association with Cf-9 on the ER membrane. A second candidate protein is a receptor-like kinase (RLK), similar to CLAVATA-1 (CLV1). The CLV perception complex determines cell fate in shoot and floral meristems of Arabidopsis and Brassica species (Torii, 2000). The CLV2 protein, which has a structure that is very similar to Cf-9, functions within a complex that includes CLV1 (Jeong et al., 1999). By analogy, Cf-9, similar to CLV2, does not contain a signalling domain and could require association with an RLK, like CLV1, to function. Because this type of RLK carries a cytoplasmic kinase domain, it may mask the dilysine motif of Cf-9.

This "masking model" could explain the results presented by Piedras et al. (2000). The CaMV 35S-driven *Cf-9* transgene likely overproduces Cf-9, without increasing expression of the masking protein. This probably results in the accumulation of Cf-9 in the ER in addition to its presence in the plasma membrane. With the use of two-phase

partitioning, Piedras et al. (2000) showed that Cf-9 is present in the plasma membrane, although the authors ignored a significant signal in the ER fraction. Moreover, labelling of protoplasts showed that Cf-9 is present at the cell surface, although the chosen experimental conditions were not suitable to detect signals in the ER. Thus, the signal in the ER that was ignored by Piedras et al. (2000) could represent an inactive form of Cf-9 that accumulates as a result of Cf-9 overexpression. Interestingly, Piedras et al. (2000) also noted that insertion of cMyc epitope tags in the G domain of Cf-9 renders the protein less active. A similar reduced activity was observed upon insertion of a FLAG epitope tag in this domain (R. A. L. Van der Hoorn, unpublished). It is likely that insertion of epitope tags in the G domain increases the distance between the membrane and the KKRY motif, which results in a less efficient masking, thereby reducing the activity of Cf-9.

Benghezal et al. (2000) used transgenic BY-2 tobacco cells containing 35S-driven *Cf-9* for two-phase partitioning to show that Cf-9 is absent from the plasma membrane fraction, whereas it is present in high amounts in the fraction containing ER membranes. It is unknown, however, whether Cf-9 is functional in these cells. Functionality of Cf-9 can be developmentally regulated (Honée et al., 1998), and the absence of the masking protein in these cells would result in complete localisation of Cf-9 to the ER. Alternatively, only a small, undetectable fraction of Cf-9 might be present in the plasma membrane of BY-2 cells, where it is functional. The authors also found endoproteolytic cleavage in the E-domain of Cf-9, which was observed only for the Cf-9-NNRY mutant and not for wild-type Cf-9 (Benghezal et al., 2000). This mutant protein is not retained in the ER and is likely exposed to proteolytic enzymes during transport to the plasma membrane. Functional Cf-9 may be resistant to these enzymes because the endoprotease recognition site can be buried in the assembled complex.

This "masking hypothesis" also could apply to Cf-4 and Hcr9-4E proteins that carry a C-terminal dilysine motif and mediate recognition of the extracellular elicitors AVR4 and AVR4E, respectively (Takken et al., 1999; Thomas et al., 1997). A plasma membrane location also is expected for Cf-2 and Cf-5, which are similar receptor-like proteins but do not contain a dilysine motif (Dixon et al., 1996; Dixon et al., 1998). These Cf proteins mediate recognition of the extracellular elicitors AVR2 and AVR5, respectively. In conclusion, all cloned tomato genes that confer resistance to the extracellular leaf pathogen *C. fulvum* encode receptor-like proteins that are likely anchored in the plasma membrane and mediate recognition of extracellular elicitor proteins. The exact process of elicitor perception on the plasma membrane is an exiting topic for further investigation.

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Chapter 6

Efficient Solubilization of the High-Affinity Binding Site for AVR9 from Tomato Membranes

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Abstract

The fungal elicitor protein AVR9 is specifically recognized by tomato plants carrying the *Cf-9* resistance gene. Recognition of AVR9 most probably requires the high-affinity binding site (HABS) for AVR9 that is present in plasma membranes of tomato and of many other plant species. Detailed characterisation of the HABS requires a solubilization procedure that does not change its binding properties for AVR9. We have developed an efficient, reproducible procedure for solubilization of the HABS, without changing its binding kinetics. Of the 19 detergents that were tested, only octyl glucoside (OG) was suitable for solubilisation of the HABS. As OG interferes with AVR9 binding, removal of OG from the solubilized fraction was required for reliable binding assays. With this procedure, the HABS remains soluble, stable and retains the same binding affinity for AVR9 as the HABS present in plasma membranes.

Introduction

The mechanism by which resistant plants can sense attacking pathogens and trigger defense responses is an intriguing topic in current research on plant-microbe interactions. In the gene-for-gene model, recognition only occurs when matching resistance *(R)* and avirulence *(Avr)* genes are present in plant and pathogen, respectively. Biochemical interpretation of the gene-for-gene model predicts that *R* gene products from the plant directly interact with *Avr* gene products from the pathogen (Gabriel and Rolfe, 1990).

The gene-for-gene pair *Avr9* and *Cf-9* has been intensively studied (Joosten and De Wit, 1999). The *Avr9* gene from the pathogenic fungus *Cladosporium fulvum* encodes a small, stable, cysteine-rich protein that is secreted into the apoplast during growth of the fungus in tomato leaves. The tomato resistance gene *Cf-9* is predicted to encode a glycoprotein that is anchored in the plasma membrane and contains a large, extracellular leucine-rich repeat (LRR) domain, which can be involved in specific protein-protein interactions. Its structure and localisation predicts a direct interaction between the AVR9 protein and the receptor-like Cf-9 protein. However, despite extensive studies using various expression systems and different types of binding assays, evidence for a direct interaction between AVR9 and Cf-9 has not been found (Luderer et al. 2001).

Nevertheless, binding assays performed with radiolabeled AVR9 (^{125}I-AVR9) showed the presence of a high-affinity binding site (HABS) for AVR9 in plasma membranes of MoneyMaker Cf9 (MM-Cf9) tomato plants (Kooman-Gersmann et al., 1996). The *Cf-9* gene itself does not encode this HABS since the same HABS is also present in MM-Cf0 tomato,

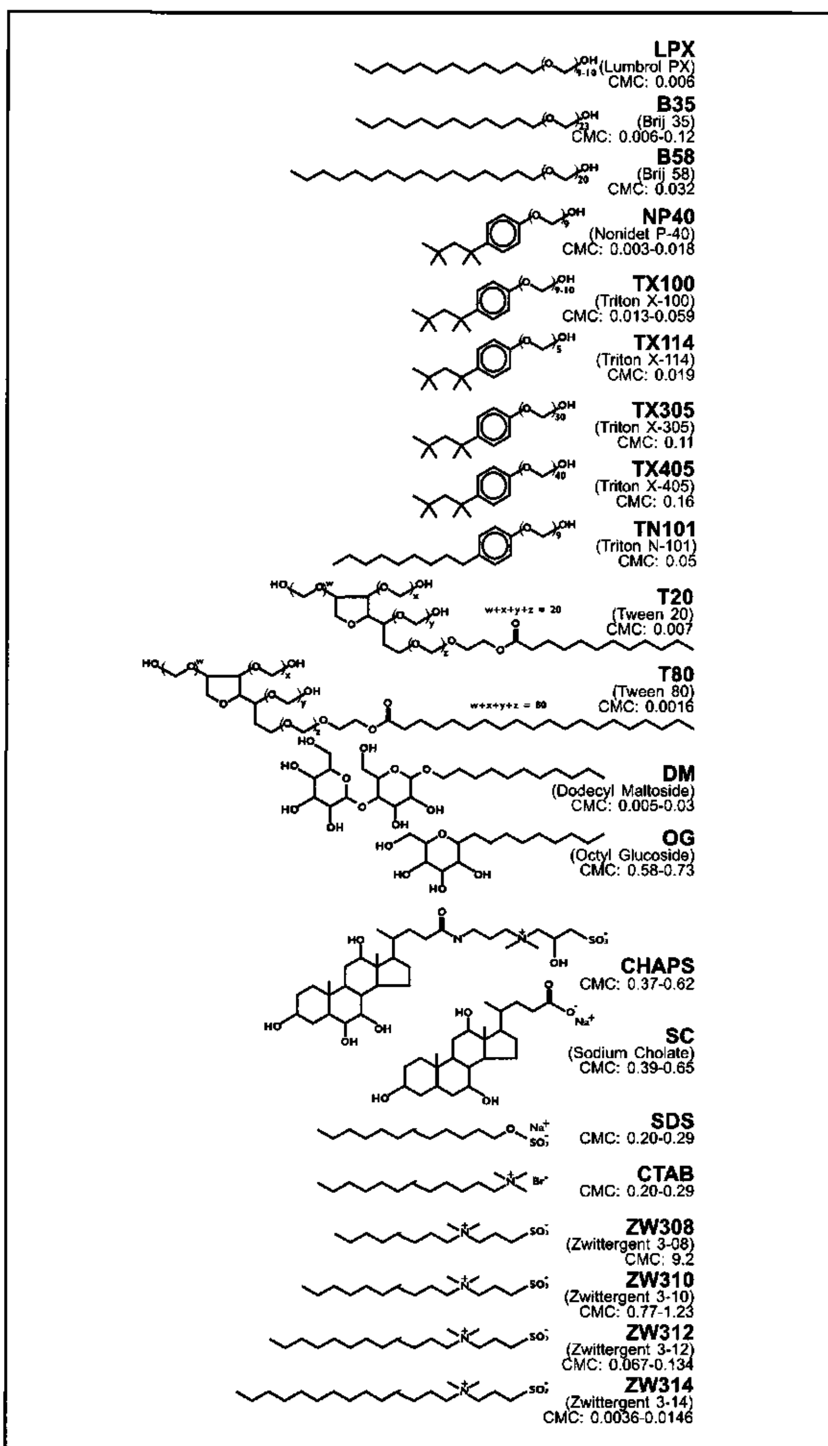
which does not carry the *Cf-9* gene. The HABS is also present in all solanaceous plant species that were tested (Kooman-Gersmann et al., 1996), and in other plant species, such as barley, oat and cucumber, but not in rice, wheat, carrot, lettuce and Arabidopsis (Kooman-Gersmann, 1998). Since the HABS is widely distributed in the plant kingdom, it likely represents a protein with a conserved function. It is difficult to imagine that the conserved function of the HABS involves recognition of AVR9, since plants different from tomato that contain the HABS are not a host for *C. fulvum*. On the other hand, maintenance of the *Avr9* gene in the fungal genome indicates that AVR9 might play a role in virulence, possibly by binding to a virulence target to suppress host defense responses or to gain nutrients from the host. Therefore, the HABS may represent the virulence target of AVR9.

Significantly, some data suggest that the HABS is also involved in recognition of AVR9 by MM-Cf9 tomato plants. Most evident are the binding experiments with mutants of AVR9, for which there is a clear correlation between the binding affinity of these mutants and their necrosis-inducing activity when injected into MM-Cf9 tomato plants (Kooman-Gersmann et al., 1998). In addition, only introduction of the *Cf-9* gene into plants that contain the HABS results in transgenic plants that are responsive towards AVR9. For example, introduction of *Cf-9* into tobacco, potato and Petunia confers responsiveness to AVR9, whereas this is not the case for Arabidopsis and lettuce (Hammond-Kosack et al., 1998; chapter 2).

The experiments described above suggest that the HABS, in addition to being a possible virulence target of AVR9, plays a key role in AVR9 perception in MM-Cf9 tomato. For further biochemical studies aimed at characterization of the HABS it is crucial that the HABS can be solubilized from the microsomal fraction (MF) without affecting its interacting abilities with AVR9.

Solubilization of membrane proteins without losing their activity depends, among others, on the detergent that is used and its concentration (Hjelmeland, 1990). Figure 1 shows the structures, trade names and critical micelle concentration (CMC) of a number of detergents that are generally used for solubilization of membrane proteins (Neugebauer, 1990; Jones et al., 1975). Below the critical micelle concentration detergent molecules only fragment the membranes, whereas above this concentration membrane proteins are effectively solubilized (Haga et al., 1990). Based on the charge of the hydrophilic side, the various detergents can be divided into three groups (Scope, 1993). Sodium cholate (SC), sodium dodecyl sulphate (SDS) and cetyl-trimethyl-ammonium bromide (CTAB) are ionic detergents and especially SDS and CTAB tend to denature proteins and are therefore not useful for solubilization of membrane proteins in their active form. The zwitterionic detergents 3-chloramido propyl-dimethyl-ammonio-1-propane sulfonate (CHAPS) and the zwittergent (ZW) series carry both positive and negative groups and are agents that are less denaturing. The remaining, non-ionic detergents have a low tendency to denature proteins. The detergents can also be grouped according to their structure (Helenius et al., 1979). Some detergents have a hydrophobic alkyl chain and a small hydrophylic head group (SDS, CTAB and ZW-series). Detergents with a hydrophilic polyoxyethylene-ether group can be subdivided in those having an alkyl chain (Lumbrol-PX and Brij's), an alkylphenyl group (Nonidet P-40 and Tritons) or a sorbitan group (Tweens). The alkylglycosides octyl

Figure 1. Structures of detergents used in this study. Trade names are given between brackets ➤ and critical micelle concentrations (CMC, in % w/v) are indicated.


LPX
(Lumbrol PX)
CMC: 0.006
B35
(Brij 35)
CMC: 0.006-0.12
B58
(Brij 58)
CMC: 0.032
NP40
(Nonidet P-40)
CMC: 0.003-0.018
TX100
(Triton X-100)
CMC: 0.013-0.059
TX114
(Triton X-114)
CMC: 0.019
TX305
(Triton X-305)
CMC: 0.11
TX405
(Triton X-405)
CMC: 0.16
TN101
(Triton N-101)
CMC: 0.05
T20
w+x+y+z = 20
(Tween 20)
CMC: 0.007
T80
w+x+y+z = 80
(Tween 80)
CMC: 0.0016
DM
(Dodecyl Maltoside)
CMC: 0.005-0.03
OG
(Octyl Glucoside)
CMC: 0.58-0.73
CHAPS
CMC: 0.37-0.62
SC
(Sodium Cholate)
CMC: 0.39-0.65
SDS
CMC: 0.20-0.29
CTAB
CMC: 0.20-0.29
ZW308
(Zwittergent 3-08)
CMC: 9.2
ZW310
(Zwittergent 3-10)
CMC: 0.77-1.23
ZW312
(Zwittergent 3-12)
CMC: 0.067-0.134
ZW314
(Zwittergent 3-14)
CMC: 0.0036-0.0146

glucoside (OG) and dodecyl maltoside (DM) consist of a hydrophilic sugar group and a hydrophobic alkyl chain. Finally, CHAPS and SC have a hydrophobic steroid structure. Due to the planar polarity, steroid detergents tend to form small micelles and dissolve membranes in into discs instead of micelles (Helenius and Simons, 1975). The other detergents have a head-tail structure and form spherical micelles that can significantly differ in size. Successful solubilization of membrane proteins in their active form has most often been achieved with CHAPS, OG, SC or TX100 (Thomas and McNamee, 1990).

In this chapter, the collection of detergents mentioned above was tested for their ability to solubilize the HABS. We present the development of a solubilization procedure that results in a reproducible, efficient solubilization of the HABS without affecting its affinity for AVR9 binding. Of the 19 detergents that were tested, only octyl glucoside (OG) was found to be suitable. However, as OG interferes with AVR9 binding, removal of OG from the solubilized fraction is required for reliable binding assays. With this procedure, the HABS remains soluble, is stable and retains the same binding affinity for AVR9 as the HABS present in microsomal fractions. This solubilisation procedure provides an excellent basis for further characterisation of the HABS and determination of its role in AVR9 perception and subsequent activation of a signal transduction cascade, leading to defence responses.

Results

Solubilisation of the HABS, followed by ^{125}I-AVR9 binding

The traditional approach to solubilize membrane proteins from a microsomal fraction (MF) is to incubate the MF with detergent for 30 minutes on ice, followed by centrifugation at 100,000g to pellet non-solubilized proteins (Figure 2A). The proteins of the supernatant (Figure 2A, fraction 4) are subsequently assayed for binding to radiolabeled ligand and the binding activity can be compared with that of the original MF (Thomas and McNamee, 1990). To solubilize the HABS from MM-Cf9 tomato MF, this approach was followed for the different detergents presented in Figure 1, at a concentration of 1% (w/v). However, with this procedure no ^{125}I-AVR9-binding activity was detected in any of the solubilized protein fractions (data not shown).

Solubilisation of the ^{125}I-AVR9-HABS complex

To test the effect of the detergent on the stability of the ^{125}I-AVR9-HABS complex, MFs were pre-incubated with ^{125}I-AVR9 to allow the formation of membrane-bound ^{125}I-AVR9-HABS complexes. These samples were subsequently treated with the various detergents (Figure 1, except SDS and CTAB) at a concentration of 1% (w/v). After incubation for 30 minutes on ice, unbound ^{125}I-AVR9 was removed and the amount of ^{125}I-AVR9 that remained bound to the HABS was measured (Figure 2B, fraction B). Many detergents (B35, B58, CHAPS, OG, SC, T20, T80, TX305, TX405 and ZW308) did not dissociate ^{125}I-AVR9 from the HABS, whereas others (DM, LPX and ZW310) dissociated about half of the bound ^{125}I-AVR9 from the HABS (Figure 3A). The remaining detergents (NP40, TN101, TX100, TX114, ZW312 and ZW314) caused substantial dissociation of the ^{125}I-AVR9-HABS complexes (Figure 3A).

The observation that some detergents do not dissociate the ^{125}I-AVR9-HABS complex, could be due to their inability to solubilize membrane proteins at the applied conditions. We therefore determined the yield of solubilized membrane proteins by incubating MFs,

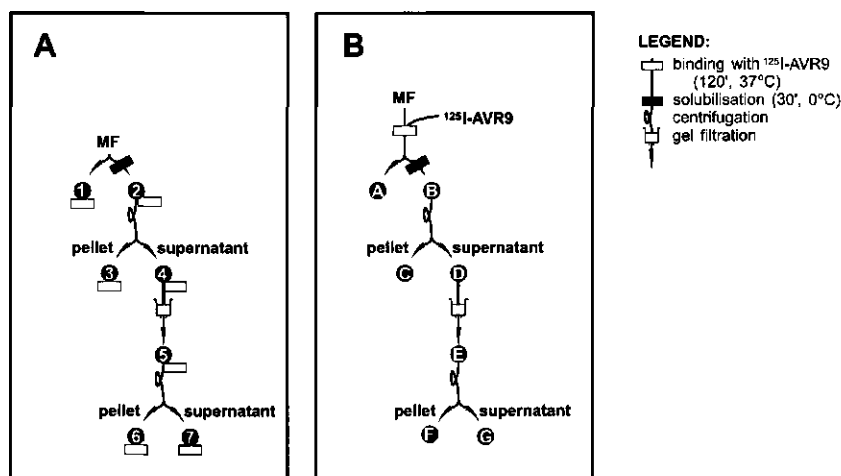


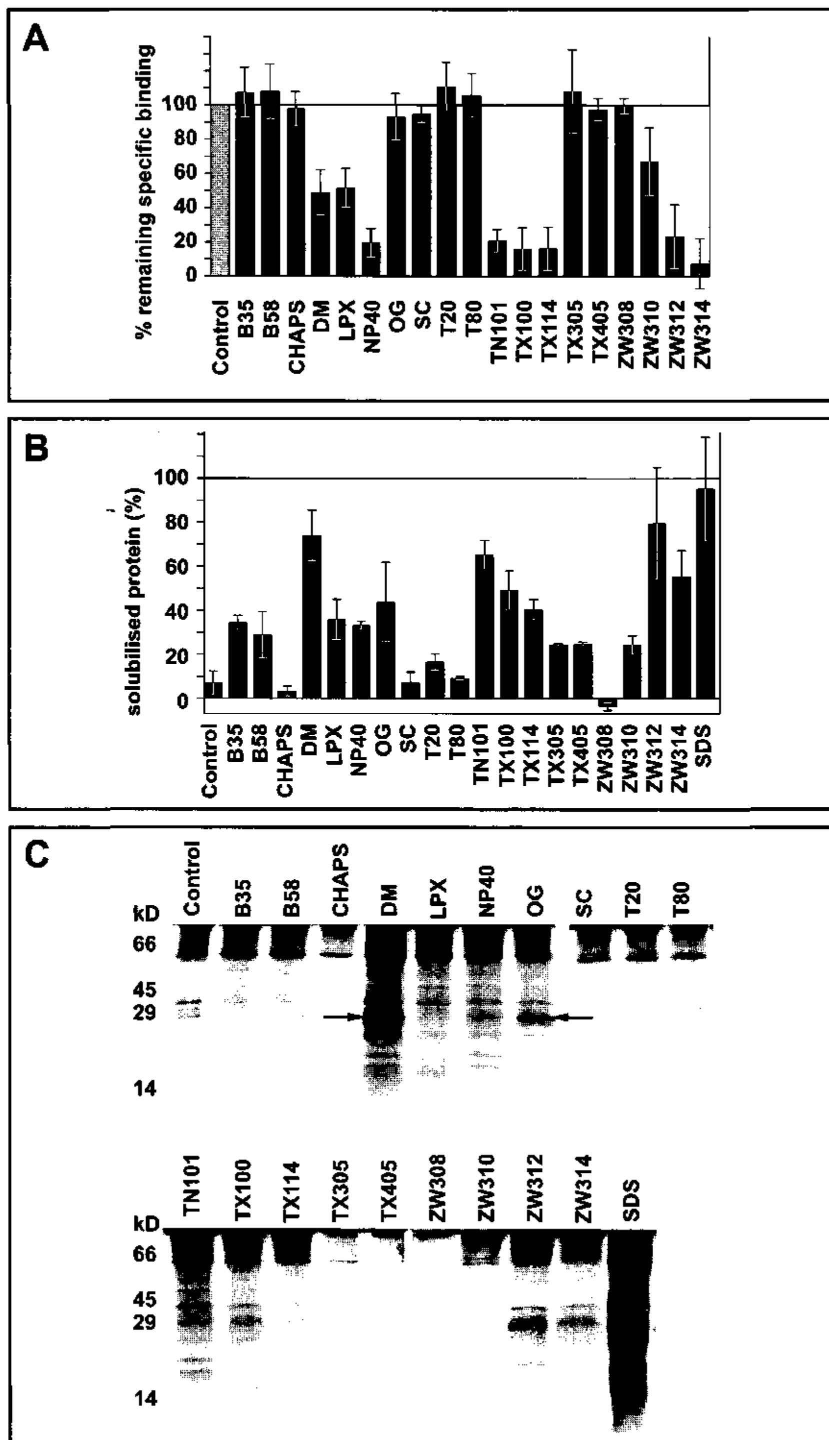
Figure 2. Solubilization procedures used in this study.

A Solubilization procedure as used for experiments presented in Figures 3B, 3C, 5 and 6. The starting material consists of microsomal fractions (MFs). During the procedure, samples 1-7 were assayed for specific binding of ^{125}I-AVR9 (see materials and methods). The centrifugation step of sample 2 was sometimes omitted from the procedure.

B Solubilization procedure as used for experiments presented in Figures 3A and 4. The starting material consists of MF, pre-incubated with ^{125}I-AVR9. Fractions (A-G) were collected after the different steps and used to measure the amount of remaining ^{125}I-AVR9-HABS complexes (see materials and methods).

without ^{125}I-AVR9, with various detergents for 30 minutes on ice. After centrifugation at 100.000g, the supernatant (Figure 2A, fraction 4) was assayed for protein concentration and composition by subjecting samples to a detergent-compatible protein assay (Figure 3B) and SDS-PAGE (Figure 3C), respectively.

Detergents that efficiently solubilize membrane proteins generally dissociate the ^{125}I-AVR9-HABS complex (compare Figures 3B and 3C with Figure 3A), suggesting that the HABS is solubilized but that the ^{125}I-AVR9-HABS complexes dissociates under these conditions. Of the detergents that partly dissociate the ^{125}I-AVR9-HABS complexes (DM, LPX and ZW310, Figure 3A), only DM is efficient in protein solubilization (Figures 3B and 3C). However, these data do not show whether the non-dissociated ^{125}I-AVR9-HABS complexes (Figure 3A) are soluble. Of the detergents that do not affect the ^{125}I-AVR9-HABS complexes (Figure 3A), the most only partly solubilize membrane proteins (Figures 3B and 3C), indicating that ^{125}I-AVR9-HABS complexes (Figure 3A) are not solubilized under these conditions and remain intact in the membrane. Only OG is able to solubilize a significant proportion of membrane proteins (Figures 3B and 3C) and keeps most of the ^{125}I-AVR9-HABS complexes intact (Figure 3A). OG therefore appears most suited to generate solubilized ^{125}I-AVR9-HABS complexes. It is interesting to note that different detergents result in different patterns of solubilized proteins (Figure 3C), which indicates that each membrane protein requires a specific detergent for efficient solubilization. For example, the protein indicated with an arrow (Figure 3C) is only efficiently solubilized by the non-ionic alkylglycosides DM and OG.


A
% remaining specific binding
100
80
60
40
20
0
Control
B35
B58
CHAPS
DM
LPX
NP40
OG
SC
T20
T80
TN101
TX100
TX114
TX305
TX405
ZW308
ZW310
ZW312
ZW314
B
solubilised protein (%)
100
80
60
40
20
0
Control
B35
B58
CHAPS
DM
LPX
NP40
OG
SC
T20
T80
TN101
TX100
TX114
TX305
TX405
ZW308
ZW310
ZW312
ZW314
SDS
C
kD
66
45
29
14
Control
B35
B58
CHAPS
DM
LPX
NP40
OG
SC
T20
T80
kD
66
45
29
14
TN101
TX100
TX114
TX305
TX405
ZW308
ZW310
ZW312
ZW314
SDS

To test whether the ^{125}I-AVR9-HABS complexes treated with non-dissociating detergents are indeed solubilized, MFs were pre-incubated with ^{125}I-AVR9, treated with the various detergents for 30 minutes on ice, centrifuged at 100,000g and the supernatant (Figure 2B, fraction D) was analysed for the presence of solubilized ^{125}I-AVR9-HABS complexes. Treatment of MFs with most of the non-dissociating detergents did result in low yields of solubilized ^{125}I-AVR9-HABS complexes (Figure 4A, black bars), as was predicted from the observation that these detergents also gave low yields of solubilized proteins (Figures 3B and 3C). Only treatment of MFs with OG resulted in solubilization of about 70% of the ^{125}I-AVR9-HABS complexes (Figure 4A).

To define why OG can solubilize the ^{125}I-AVR9-HABS complexes, whereas the traditional procedure in which membrane proteins are first solubilized and subsequently analysed for binding, was unsuccessful, we focused on the stability of the solubilized ^{125}I-AVR9-HABS complexes, either in the presence or absence of OG. OG-solubilized ^{125}I-AVR9-HABS complexes (Figure 2B, fraction D), were incubated at various temperatures and samples were taken to determine the amount of remaining ^{125}I-AVR9-HABS complexes. At 4°C, the ^{125}I-AVR9-HABS complexes remain stable for several hours (Figure 4B), whereas at room temperature (20°C) the ^{125}I-AVR9-HABS complexes dissociate within 1.5 hours. At 37°C, the ^{125}I-AVR9-HABS complexes are completely dissociated within half an hour (Figure 4B). Lack of binding of ^{125}I-AVR9 to the HABS, solubilized by pre-treatment with OG (Figure 2A, fraction 4), is therefore likely due to the presence of OG in these protein fractions. In the assay shown in Figure 2A, ^{125}I-AVR9 binding is performed by incubation of the OG-solubilized fraction with ^{125}I-AVR9 for two hours at 37°C. The presence of 1% OG will result in dissociation of any formed ^{125}I-AVR9-HABS complexes, or even inhibit any interaction between ^{125}I-AVR9 and the HABS.

Solubilization of the HABS

During one of the solubilization experiments in which OG was used, it was noted that the rate of dissociation of the solubilized ^{125}I-AVR9-HABS complexes decreased by dilution of the solubilized fraction in a buffer without OG (data not shown). This, together with the observations described in the previous section, suggested that removal of OG might increase the stability of the solubilized ^{125}I-AVR9-HABS complexes. Therefore, OG was removed from the OG-solubilized fraction (Figure 2B, fraction D) by gel filtration over Sephadex G200 (see materials and methods). The ^{125}I-AVR9-HABS complexes were present in the void volume of

Figure 3. Effect of the various detergents on the stability of the ^{125}I-AVR9-HABS complexes and protein solubilization.

A Effect of detergents on the stability of the ^{125}I-AVR9-HABS complexes.
Membranes containing ^{125}I-AVR9-HABS complexes were treated with the various detergents at a concentration of 1% (w/v). Remaining specific binding was measured in fractions B (Figure 2B) and compared to membranes that were incubated in buffer without detergent (control).

B and **C**, Solubilization of membrane proteins from MFs with different detergents. The solubilized protein fraction was obtained after treatment with different detergents at 1% (w/v) (Figure 2A, fraction 4).

B The protein concentration of the supernatant was determined using a detergent-compatible assay and compared to the initial protein concentration of MF.

C Proteins present in the supernatant were separated by SDS-PAGE. The arrow indicates a protein that is most efficiently solubilized by DM and OG.

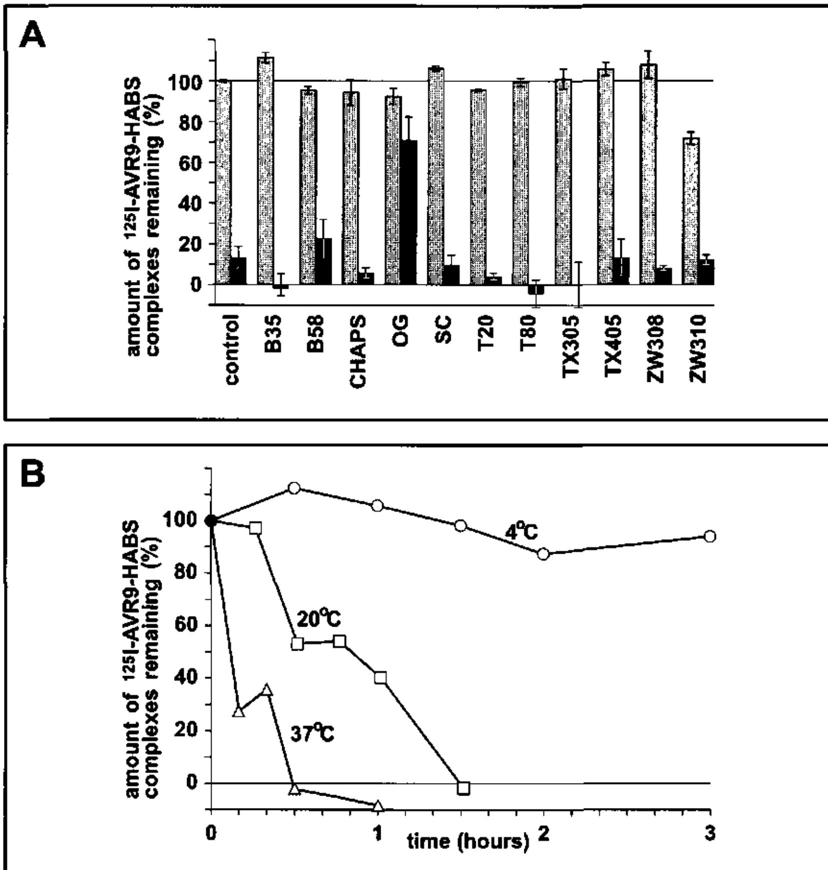


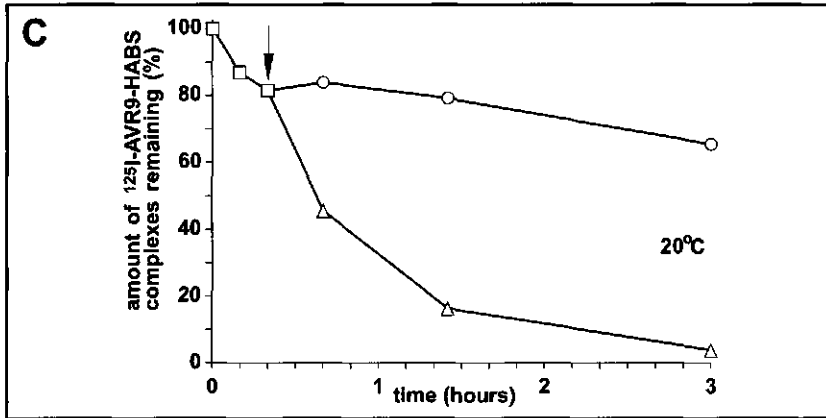
Figure 4. Solubilization and stability of the solubilized ^{125}I-AVR9-HABS complexes.

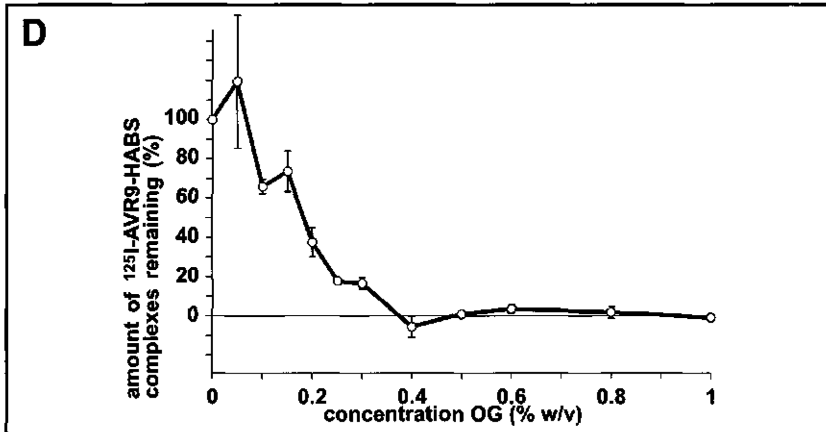
A Solubilisation of ^{125}I-AVR9-HABS complexes. MFs containing ^{125}I-AVR9-HABS complexes were treated with different detergents at a concentration of 1% w/v and centrifuged at 100,000g. Specific binding of ^{125}I-AVR9 was determined before centrifugation (grey bars, fraction B in Figure 2B) and in the supernatant (black bars, fraction D in Figure 2B), and compared to specific binding of untreated MF (control).

B Stability of the ^{125}I-AVR9-HABS complex in the presence of 1% (w/v) OG. OG-solubilized ^{125}I-AVR9-HABS complexes (Figure 2B, fraction D) were incubated at 4°C, 20°C and 37°C and samples were taken at various time points. The remaining amount of specific binding was compared to the initial specific binding. The assay was repeated twice, with similar results.

C Removal of OG stabilises ^{125}I-AVR9-HABS complexes. OG was removed from OG-solubilized membrane proteins by gel filtration and the amount of ^{125}I-AVR9-HABS complexes present in the void volume (Figure 2B, fraction E) was compared to initial specific binding. At 20°C, the ^{125}I-AVR9-HABS complex remains stable for several hours (circles), whereas addition of OG to a final concentration of 1% w/v (arrow) results in a quick and almost complete dissociation of the ^{125}I-AVR9-HABS complexes (triangles). The assay was repeated twice, with similar results.

D Effect of OG concentration on ^{125}I-AVR9 binding. MFs were incubated with ^{125}I-AVR9 and different concentrations of OG. After incubation for two hours at 37°C, specific binding was determined and compared to that of samples without detergent.





the column (Figure 2B, fraction E, squares in Figure 4C). As gel filtration also resulted in removal of unbound ^{125}I-AVR9, the initial decrease of the amount of ^{125}I-AVR9-HABS complexes (squares in Figure 4C) could result from a resettlement of the equilibrium between free ^{125}I-AVR9, and ^{125}I-AVR9 bound to the HABS. Significantly, ^{125}I-AVR9-HABS complexes in this fraction remained stable for several hours at room temperature (20°C, circles in Figure 4C). To examine whether OG is indeed responsible for the dissociation of ^{125}I-AVR9-HABS complexes, OG was added to a final concentration of 1% (w/v) (arrow in Figure 4C). This resulted in a complete dissociation of the ^{125}I-AVR9-HABS complexes (triangles in Figure 4C), with similar kinetics as was observed before gel filtration (Figure 4B). To test whether the ^{125}I-AVR9-HABS complexes were still solubilized after removal of OG by gel filtration, the void volume was centrifuged at 100,000g. Most of the ^{125}I-AVR9-HABS complexes remained in the supernatant, demonstrating that the ^{125}I-AVR9-HABS complexes were still solubilized (data not shown). Thus, 1% (w/v) OG is responsible for dissociation of ^{125}I-AVR9-HABS complexes and its presence is not required to keep ^{125}I-AVR9-HABS complexes in solution.

To determine the maximal concentration of OG that is allowed in binding assays, MFs were incubated with ^{125}I-AVR9 in combination with different concentrations of OG. After incubation for 2 hours at 37°C, samples were taken to determine the amount of ^{125}I-AVR9-HABS

complexes (Figure 2B, fraction 2). Figure 4D shows that the presence of more than 0.2% (w/v) OG in the binding assay inhibits the formation of ^{125}I-AVR9-HABS complexes. Concentrations below 0.1% (w/v) OG had no influence of ^{125}I-AVR9 binding to the HABS (Figure 4D).

The observation that OG can be used to solubilize the ^{125}I-AVR9-HABS complex, and that the ^{125}I-AVR9-HABS complex remains in solution after removal of the OG, allows ^{125}I-AVR9 binding to OG-solubilized fractions after removal of OG. We therefore included a gel filtration step after solubilization with OG (Figure 2A). Samples were taken at all steps in the solubilization procedure and used in a standard binding assay with ^{125}I-AVR9 (Figures 2A and 5, fractions 1-7). We now found specific binding in the void volume after gelfiltration (Figure 5, fraction 5) and this binding activity remained in the supernatant after centrifugation (Figure 5, fraction 7). The absence of specific binding in fractions 2 and 4 (Figure 5) is probably due to the presence of 1% (w/v) OG in these fractions, whereas specific binding in the pellets (Figure 5, fractions 3 and 6) was observed since they were resuspended in a buffer without OG and contain non-solubilized HABS. Nine independent solubilization experiments showed that this procedure results in solubilization of 78 ± 35% of the total amount of HABS that are present in the MF, whilst 26 ± 8% of the total membrane proteins are solubilized. Thus, apart from being reproducible and efficient, this approach also results in a three-fold purification of the HABS.

Characterization of the solubilized HABS

To further characterize the OG-solubilized HABS after gel filtration (Figure 2A, fraction 7), binding of ^{125}I-AVR9 at 37°C to the solubilized HABS was followed in time. Figure 6A shows that binding of ^{125}I-AVR9 reaches equilibrium within two hours. This binding is reversible, since addition of a 1000-fold excess of unlabeled AVR9 (at t=1hr, arrow in Figure 6A) results in a rapid decrease of ^{125}I-AVR9 binding (triangles in Figure 6A). Eventually the amount of bound ^{125}I-AVR9 reaches a level similar to the level reached when a 1000-fold excess of unlabeled AVR9 and ^{125}I-AVR9 is added simultaneously at t=0 hr (squares in Figure 6A). The kinetics of ^{125}I-AVR9 binding to the solubilized HABS is similar to that observed for the HABS present in MF (Van der Hoorn, unpublished).

Incubation of the solubilized HABS (Figure 2A, fraction 7) with increasing concentrations of ^{125}I-AVR9 revealed that the binding is saturable (Figure 6B). Transformation of these data into a Scatchard plot (Figure 6C) yielded an apparent K_D of 0.14 ± 0.07 nM (n=9). This is similar to the K_D that was found for the HABS present in MF (K_D = 0.07 nM, Kooman-Gersmann et al., 1996). Therefore we conclude that solubilization of the HABS does not change its affinity for AVR9.

Discussion

Solubilization of the HABS for AVR9 without affecting its function is a crucial prerequisite for further studies on the mechanism by which AVR9 is perceived by resistant tomato plants. Furthermore this approach may also uncover the role of the HABS as virulence target of AVR9. Here, we have developed an efficient and reproducible solubilization procedure for the HABS, without affecting its binding kinetics.

From the 19 detergents that were tested for solubilization of ^{125}I-AVR9-HABS complexes from microsomal fractions (MFs) of MM-Cf9 tomato plants, only octyl glycoside

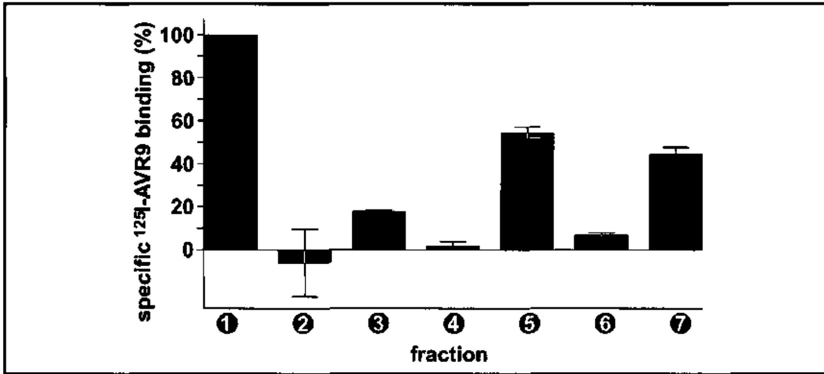


Figure 5. Solubilization of the HABS
Proteins present in MF were solubilized as shown in Figure 2A and samples of fractions 1-7 were taken for a binding assays with ^{125}I-AVR9. Specific binding was determined compared to that of the original MF (fraction 1).

(OG) proved to be suitable. Efficient and reproducible solubilization of ^{125}I-AVR9-HABS complexes by OG did not require a further screen for solubilization by other detergents at different concentrations. However, we cannot exclude that solubilization of ^{125}I-AVR9-HABS complexes from MFs under other conditions can also be successful. For example, dodecyl maltoside (DM) is structurally similar to OG and was effective in protein solubilization, but also dissociated half of the ^{125}I-AVR9-HABS complexes. Possibly at a lower DM concentration dissociation of the ^{125}I-AVR9-HABS complexes would not occur, whilst the intact complexes are still solubilized. Similarly, the detergents NP40, TN101, TX100, TX114 and ZW312 are also efficient in protein solubilization, but dissociate ^{125}I-AVR9 from the HABS. Also in this case, their low CMC could still allow efficient solubilization of ^{125}I-AVR9-HABS complexes at lower detergent concentrations, which may not dissociate the ^{125}I-AVR9-HABS complexes. The relation between CMC values and solubilization efficiency is most evident in the Triton- and ZW-series. Detergents with a relatively high CMC (TX405 and ZW308) solubilize only small amounts of proteins and keep the ^{125}I-AVR9-HABS complex intact, whereas detergents with a relatively low CMC (TX100 and ZW312) are efficient in solubilization of membrane proteins but dissociate the ^{125}I-AVR9-HABS complex. Other detergents that are frequently used in solubilization procedures, such as B35, CHAPS and SC, did not dissociate the ^{125}I-AVR9-HABS complexes, but also solubilized only small amounts of membrane proteins. In case of CHAPS, SC and ZW308, solubilization of membrane proteins did probably not occur due to the relatively high CMC of these detergents.

Although we did not test many different solubilization conditions, it is likely that ^{125}I-AVR9-HABS complexes require specific detergents for efficient solubilization. Solubilization conditions for a membrane protein in its active form can vary significantly for different proteins. For example, OG did not solubilize the binding site for the hepta ß-glucoside elicitor from soybean membranes, whereas DM and ZW312 allowed solubilization up to 54% and 40% of the binding activity, respectively (Cosio et al., 1990). In contrast, 91% of the fusicoccin-binding activity could be solubilized from oat membranes by OG, but CHAPS, LPX, SC, TX100 or ZW314 were less efficient (De Boer et al., 1989). Furthermore, solubilization of glutamate-

binding activity from membranes from insect cells was effective when TX100, TX114 or OG were used, but was less for the steroid-derivatives CHAPS and SC (Kuusinen et al., 1995). In contrast, the adenosine-binding activity from rat brain membranes was solubilized with SC and CHAPS with 30-35% efficiency, whilst TX100, LPX, B35 and T80 were not effective (Nakata and Fujisawa, 1983). Finally, B35, CHAPS, OG and TX100 were effective in solubilization of transglutaminase activity from sugar beet membranes (Signorini, 1991). Thus, the choice of detergent appears to depend strongly on the protein of interest and cannot be predicted. In our studies, the different solubilisation behaviour of membrane proteins upon treatment of MFs with various detergents was also clear from the protein patterns on SDS-PAGE (Figure 3C). In addition, the three-fold purification of the HABS for AVR9 during solubilization of proteins present in MFs with OG indicates that OG solubilizes different proteins with different yields.

A crucial step in the solubilization procedure of the HABS in its active form is gel filtration, which is used to remove excess detergent from the solubilized protein fraction. Since free detergent molecules are in equilibrium with micelles at concentrations above the CMC, micelles are expected to gradually disappear during gel filtration, as free detergent molecules are retained in the column matrix. Probably only detergent molecules that strongly interact with high molecular weight proteins are not retained by the gel filtration column. Remaining detergent may explain the observation that many membrane proteins, including the HABS, remain soluble after gel filtration. The concentration of OG that remained after gel filtration could not be determined. However, binding assays indicated that it must have been below 0.1% (w/v), since concentrations above this value severely inhibit binding between ^{125}I-AVR9 and the HABS (Figure 4D). The dissociating effect of OG on the ^{125}I-AVR9-HABS interaction was most evident after addition of OG after gel filtration of the solubilized membrane proteins (Figure 4C). The inhibitory effect of high concentrations of OG on ^{125}I-AVR9 binding is probably largely due to the fact that binding of ^{125}I-AVR9 requires incubation at high temperature (37°C). A similar inhibitory effect was observed for fusicoccin binding at 30°C (De Boer et al., 1989). Binding assays with other ligands are usually performed at 4°C or room temperature, in which case effects of OG on binding will probably not be observed.

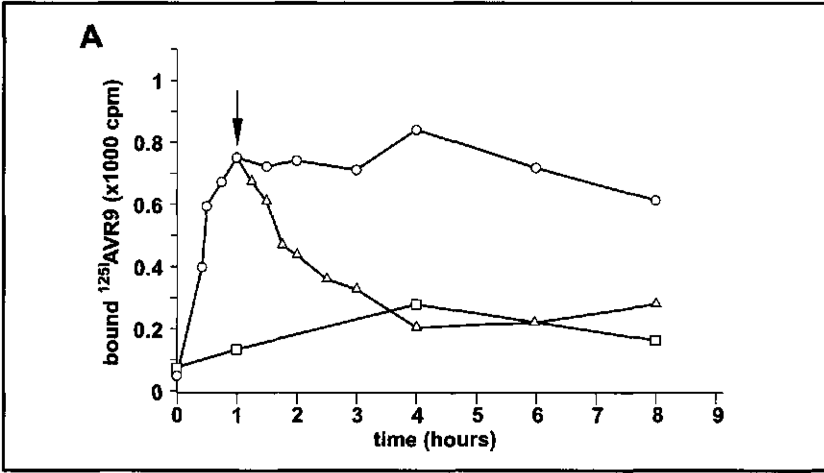
The solubilization protocol for the HABS of AVR9 allows its further characterisation. One approach will be to purify the solubilized HABS on an AVR9-affinity column and to subsequently characterise the purified protein(s) by sequencing. However, despite extensive efforts to purify the HABS in this way, we have not been successful thus far, probably because immobilized AVR9 loses its affinity for the solubilized HABS (R. Van der Hoorn, unpublished). However, a combination of derivatives of AVR9, different affinity matrices and avidin-biotin technology may be used to circumvent this problem.

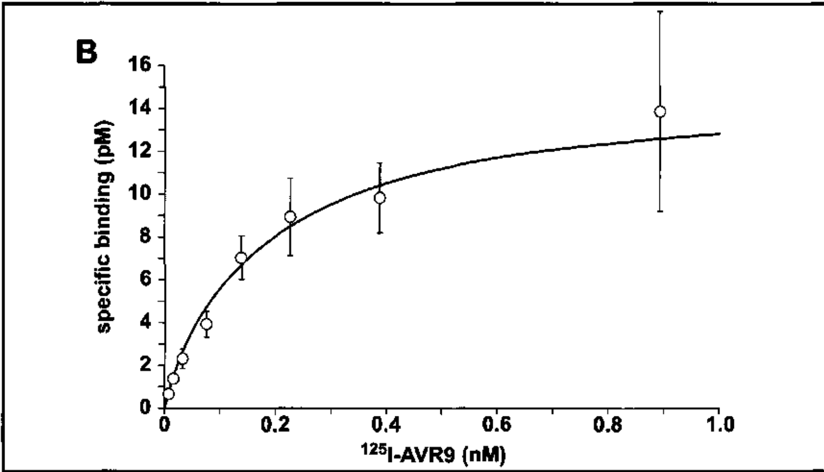
Figure 6. Characterization of the solubilized HABS.

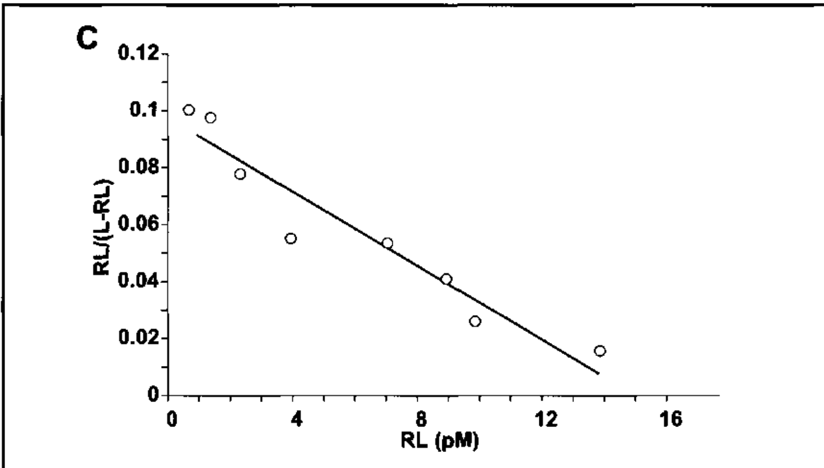
A Time course experiment of ^{125}I-AVR9 binding to the OG-solubilized HABS. The solubilized fraction, obtained as described in Figure 2A (fraction 7), was incubated with ^{125}I-AVR9 at 37°C and binding was measured in time (circles). At t = 1hr (arrow), a 1000-fold excess unlabeled AVR9 was added to show the reversibility of the interaction (triangles). Squares represent non-specific ^{125}I-AVR9 binding in the presence of a 1000-fold excess of unlabeled AVR9 added at t = 0. The assay was repeated two times, giving similar results.

B Saturation of ^{125}I-AVR9 binding to the solubilized HABS. The solubilized fraction, obtained as described in Figure 2A (fraction 7), was incubated with different concentrations of ^{125}I-AVR9 for two hours at 37°C.

C Scatchard plot of data obtained from Figure 6B.


A
bound 125I-AVR9 (x1000 cpm)
0
0.2
0.4
0.6
0.8
1
0
1
2
3
4
5
6
7
8
9
time (hours)


B
specific binding (pM)
0
2
4
6
8
10
12
14
16
0
0.2
0.4
0.6
0.8
1.0
125I-AVR9 (nM)


C
RL/(L-RL)
0
0.02
0.04
0.06
0.08
0.1
0.12
0
4
8
12
16
RL (pM)

Materials and Methods

Materials

OG and SC were from Boehringer-Mannheim (Mannheim, Germany); SC and the ZW-series from Calbiochem (La Jolla, CA, USA); B35, B38, CHAPS, NP40, TX305 and TX405 from Pierce (Rockford, IL, USA); TN101 from Fluka (Zwijndrecht, The Netherlands); T20 and T80 from Merck (Darmstadt, Germany) and DM, LPX and TX100 from Sigma-Aldrich (St. Louis, MO, USA). ^{125}I-AVR9 was prepared as described previously (Kooman-Germann et al., 1996). The AVR9 peptide was synthesised according to the method described by Mahé et al. (1998) and Van den Hooven et al. (1999).

Preparation of microsomal fractions

Leaves from the tomato genotype MM-Cf9 were harvested from 4-6-week-old plants that were grown under normal greenhouse conditions. Microsomal fraction (MF) was isolated by grinding the leaves in a blender for 4 minutes at 4°C in ice-cold MB1 buffer (25mM Tris-HCl, pH 7.5, 250mM sucrose, 3mM EDTA). Following filtration through two layers of miracloth (Calbiochem, La Jolla, CA), debris was removed by centrifugation for 20 minutes at 10.000g (Sorvall RC5C centrifuge, Dupond). The supernatant was subsequently centrifuged for 40 minutes at 100.000g (Beckman L7-65 ultracentrifuge) and the pellet was resuspended in SB2 buffer till a final concentration of 10-20 mg/ml (25mM sodium-phosphate, pH 6, 250mM sucrose). The resulting MF preparations were stored at -80°C.

Solubilization

Detergents were dissolved in SB2 at 2% (w/v). After adding this to an equal volume of MF, the samples were incubated on ice for 30 minutes. Samples were subsequently centrifuged in an ultracentrifuge (40 min. 100.000g, 4°C, Beckman L7-65 ultracentrifuge), and the supernatant was taken for further experiments.

SDS-PAGE

Proteins were separated on 10% polyacrylamide gels containing SDS and subsequently stained with Coomassie Brilliant Blue. Protein concentrations were measured by the Lowry assay (Detergent Compatible Protein Assay, BioRad, Hercules, CA, USA)

Gel filtration

Custom disposable gel filtration columns were made by pouring Sephadex G200 (Pharmacia Biotech, Uppsala, Sweden) into a 2ml syringe, using glass wool as a grid. After packing of the gel matrix, the column was equilibrated with 10ml SB2. 400µl samples were added to the columns after which the void volume was collected upon addition of 600µl of SB2 to the column. This type of gel filtration was also used to remove unbound ^{125}I-AVR9 from fractions containing solubilized proteins.

Binding assays using ^{125}I-AVR9

Samples containing 5-50µg proteins were mixed with ^{125}I-AVR9 in a total volume of 100µl SB2. Aspecific binding was determined in the presence of a 1000-fold excess of unlabeled AVR9, whereas total binding was determined in the absence of unlabeled AVR9. After incubation at 37°C, unbound ^{125}I-AVR9 was removed either by gel filtration or filtration through a glass filter. These separation methods gave similar results. In the case of gel filtration, the void volume containing bound ^{125}I-AVR9 was collected (see above) and its radioactivity was determined. In the case of filtration through glass filters, glass fiber filters (GF6, Schleicher & Schuell, Den Bosch, The Netherlands), were incubated for at least 1 hour in 0.5% polyethylenimine (Sigma-Aldrich, St. Louis, MO, USA), transferred to a filtration manifold (Millipore, Bedford, MA), and washed with 5ml SB2. After filtration of the samples, the filters were washed two times with 5ml SB2 and radioactivity on the filters was determined. For measuring radioactivity, gel filtration fractions or glass filters were transferred to scintillation vials and 3ml of LumaSafe Plus (LUMAC.LSC B.V. Groningen, The Netherlands) were added. The radioactivity was counted in a scintillation counter (model LS-6000 TA, Beckman Instruments). Specific binding was calculated by subtracting the aspecific binding from the total binding. The concentration of ^{125}I-AVR9 was usually 10^{-10} M and the incubation time was usually 2 hours at 37°C, unless indicated otherwise. Binding assays were performed in triplicate.

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Chapter 7

General Discussion

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Modified version submitted for publication

General Discussion

'Boom-and-bust' cycles in agriculture

In agriculture, significant losses of crops due to diseases frequently occur. Chemicals are often used to prevent diseases or cure diseased plants, but often have side effects that are harmful to the ecosystem. Traditionally, resistant plant varieties are regularly used to suppress pathogen epidemics. In most cases, resistance has been introgressed into crop species from wild relatives by classical breeding programs. Once released on the market, these new varieties become popular, resulting in 'booming' of the area planted with these new varieties. However, in many cases, these varieties became also infected, since most pathogens are eventually able to overcome host resistance. Large areas sown or planted with the new variety become susceptible and outbreaks of large pathogen epidemics occur. Subsequently, the plant variety becomes less popular, resulting in 'busting' of this plant variety. To cope with the new, virulent strain of the pathogen, a new resistance trait has to be introgressed into the crop species. For many plant-pathogen interactions, these 'boom-and-bust' cycles are already ongoing for decades. The continuous introduction of new resistance genes into crop species, followed by the emergence of new virulent strains of the pathogen, explains why this classical resistance breeding can be an expensive, time-consuming and often inefficient process to suppress diseases. In nature, plants have survived pathogen attack for millions of years. By examining mechanisms of natural disease resistance in detail, we might understand how natural plant populations have learned to cope with pathogens. With this knowledge it should be possible to improve protection of crop plants employed in modern agriculture.

The discovery of 'gene-for-gene' interactions between plants and pathogens

'Boom-and-bust' cycles forced breeders to introduce a large set of resistance genes into crop plants. It is known that disease resistance is usually a monogenic, dominant trait. In many cases, these 'resistance genes' only confer protection against particular strains of the pathogen, which are then called 'avirulent'. Within the pathogen, avirulence is also often found to be a monogenic, dominant trait. These observations led to the 'gene-for-gene' hypothesis, which states that for every dominant resistance (*R*) gene in the plant, there is a corresponding dominant avirulence (*Avr*) gene in the pathogen (Flor, 1942). Thus, gene-

Table 1: Gene-for-gene interactions between plants and pathogens

plant genotype	pathogen genotype		
	R1, r2, r3	*r1, R2, r3*	*r1, r2, R3*
A1, a2, a3	I	C	C
a1, A2, a3	C	I	C
a1, a2, A3	C	C	I

Outcome of interactions between plants with different *R* genes (top) and pathogens with different *Avr* (*A*) genes (left). I, Incompatible interaction: pathogen is avirulent and plant is resistant. C, Compatible interaction: pathogen is virulent and plant is susceptible. For simplicity only one allele of each gene is represented.

for-gene interactions can be schematically represented by an interaction scheme with plant genotypes carrying different *R* genes on one side, and pathogen strains carrying different *Avr* genes on the other side (Table 1).

Gene-for-gene relations as a consequence of an 'arms-race'

Recurrent 'boom-and-bust' cycles resulted in multiple gene-for-gene interactions between crop plants and their pathogens. Gene-for-gene interactions also exist in natural ecosystems. In order to survive in nature, plant species have to be able to generate new resistance specificities to cope with fast-adapting pathogens (Figure 1). The ongoing co-evolution between plants that develop new resistance specificities and pathogens that try to circumvent these new recognitional specificities, can be seen as an 'arms-race' between plants and pathogens, that is driven by selection pressure (Dawkins and Krebs, 1979). Thus, gene-for-gene interactions are the result of an 'arms-race' between plants and pathogens, whereas 'boom-and-bust' cycles in modern agriculture represent an artificial simulation of the 'arms-race' in natural populations.

Recognitional specificities of *R* gene products

The simplest biochemical interpretation of the gene-for-gene interaction is that an *Avr* gene encodes a ligand that specifically interacts with a receptor encoded by the matching *R* gene (Keen, 1990). Consistent with this theory, it was found that *R* genes encode receptor-like proteins that belong to only a few different families (Table 1 of chapter 1). Resistance gene *Cf-9* of tomato, which confers recognition of the fungus *C. fulvum* carrying the *Avr9* gene, is the founder member of a large class of *R* genes that encode membrane-anchored proteins containing a transmembrane domain (TM) and extracytoplasmic leucine-rich repeats (LRRs) (LRR-TM class). The other major class of *R* genes encodes cytoplasmic proteins that contain LRRs and a nucleotide-binding site (NBS) (NBS-LRR class). Within the LRR domain, solvent-exposed amino acid residues are expected to form the recognitional surface that decorates the parallel ß-sheet plane on one side of these proteins. Indeed, by domain-swap analysis between *Cf-4* and *Cf-9*, we could confirm that amino acid residues at putative solvent-exposed positions play a crucial role in determining the specificity in resistance proteins (chapter 3). However, the role of these residues in mediating recognition of the corresponding avirulence protein remains to be elucidated.

Sequence exchange and diversifying selection as mechanisms to generate *R* genes with new recognitional specificities

Generation of new recognitional specificities is thought to involve two main mechanisms (Parniske et al., 1997; Michelmore and Meyers, 1998). These are: sequence exchange between homologous genes, and diversifying selection of solvent-exposed amino acid residues in the LRR domain. The observation that clustered family members of *R* genes consist of sequence patchworks led to the suggestion of the sequence exchange mechanism. Diversifying selection was suggested by the observation that *R* gene family members predominantly differ at amino acid residues at putative solvent-exposed positions, and that non-synonymous nucleotide substitutions in their corresponding codons occur more frequently than synonymous substitutions. These observations were first reported for *Cf-9* homologs located at the *Cf-9* and *Cf-4* gene clusters by Parniske et al. (1997), and later confirmed for other *R* gene families (Noël et al., 1999; Van der

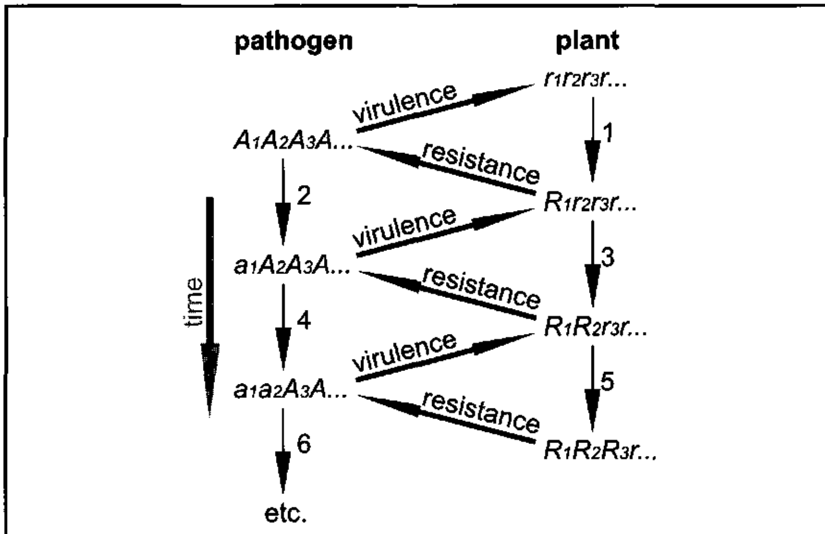

Figure 1. The 'arms-race' between plants and pathogens results in multiple, complex gene-for-gene relations. The pathogen uses various virulence genes (*A*, see below) to attack a susceptible plant. As a result of selection pressure, in step 1 the plant develops an *R* gene (*R1*) that confers recognition of specific virulence gene product A1 of the pathogen, resulting in resistance. As a consequence, the *A1* gene is now referred to as an *Avr* gene. In step 2, as a result of selection pressure that is now imposed on the pathogen, the pathogen circumvents this recognition by a mutation in the *A1* gene, rendering the plant that carries the *R1* gene susceptible. In time, these cycles continue, eventually resulting in the multiple, complex gene-for-gene relations as we see them now.

Vossen et al., 2000; Botella et al., 1998; Ellis et al., 1999; Meyers et al., 1998; McDowell et al., 1998 and Song et al., 1997).

In chapter 3, three amino acids at putative solvent-exposed positions were identified that contribute to Cf-4 specificity. Two of these are not present in the other Cf homologs that have been described. This suggests that diversifying selection at solvent-exposed positions has been an important factor to generate Cf-4 specificity. Domain swaps between Cf-9 and Cf-4 revealed that most of the swaps do not affect Cf-4 function. Also analysis of the *9DC* gene of *L. pimpinellifolium* (chapter 4) revealed that extensive sequence exchange between *Cf* genes may not necessarily result in altered specificity. Therefore, the role of sequence exchange between *Cf* homologs in the generation of *Cf* genes with new specificities remains to be proven. However, in the NBS-LRR gene family, a significant role for sequence exchange in the generation of new specificities has been shown for the *L* genes in Flax (Ellis et al., 1999). Sequence exchange has also been suggested to be the basis of the polymorphism found between the *HRT* and *RPP8* genes of *Arabidopsis thaliana* (Cooley et al., 2000).

Cf genes are generated through 'birth-and-death' evolution, rather than through 'adaptive evolution'

As described above, sequence exchange and diversifying selection are likely the two main mechanisms that create *R* genes with new specificities. At a higher level, there are two

different theories concerning the evolution of an *R* gene with a particular recognitional specificity. In adaptive evolution, it is expected that once a particular gene confers recognition of an avirulence determinant, even with weak affinity, it becomes adapted to this function by the two mechanisms mentioned above (Richter and Ronald, 2000). As a result of this 'adaptive evolution', most specific features of the resistance protein are required for optimal recognition of the corresponding avirulence determinant. In contrast, according to the 'birth-and-death' model, an *R* gene with a particular specificity is 'born' spontaneously by the two mechanisms mentioned above (Michelmore and Meyers, 1998). Once born, this R protein is not forced to evolve further. A similar spontaneous event may result in a sudden 'death' of this *R* gene. According to this 'birth-and-death' model, most specific features in the R protein are accidentally present and do not play an essential role in recognition of the corresponding Avr determinant. In both models, maintenance of the *R* gene in the population will depend on selection pressure.

A striking observation is that most variation in functional Cf proteins is not required for its recognitional specificity. We have shown this for Cf-4 function, for which we constructed a Cf-9 mutant that confers AVR4 recognition (chapter 3). A similar observation was made for the 9DC protein, which is very different from Cf-9 but also confers recognition of AVR9 (chapter 4). Thus, significant variation in Cf proteins with identical recognitional specificity has not only been found for artificial mutants generated in the laboratory, but also occurs in nature. Therefore, Cf proteins can be seen as scaffolds with flexible decorations. These data fully comply with the 'birth-and-death' model, rather than 'adaptive evolution' of *R* genes. The variation that is present in the decoration of Cf proteins may represent a versatile pool for the generation of new specificities.

Gene-for-gene interactions in nature are maintained through 'trench-warfare' between plants and their pathogens

The high rate of pathogen adaptation that we know from 'boom-and-bust' cycles in modern agriculture suggests that similar dynamics also occur in nature. Therefore, the generation of *R* genes with new specificities is expected to be a relatively quick process. Indeed, some *R* gene clusters, like the *Rp1* cluster in maize (Hulbert and Bennetzen, 1991), are genetically unstable, which was often explained by the presence of multiple *R* gene homologs that stimulate unequal crossing-over and other events, possibly leading to generation of *R* genes with new specificities. However, recombination between genes at other *R* gene clusters, like *Rps2* in Arabidopsis, *Dm3* in lettuce and *Mi* and *Tm2a* in tomato (Chin et al., 2001; Wei et al., 1999; Van Daelen et al., 1993 and Ganal et al., 1989), appears to be suppressed. Significantly, the existence of *RPM1* and *Pto* genes in different species of *Arabidopsis* and *Lycopersicon*, respectively, suggests that *R* genes with particular specificities already existed before the species diverged (Stahl et al., 1999: Riely and Martin, 2001). It was also noted that plants carrying *RPM1* co-exist with plants that lack this gene (Stahl et al., 1999). Alltogether, this supports the hypothesis that in nature, gene-for-gene pairs are maintained over a long period of time as a result of 'trench-warfare' between plants and pathogens (Stahl et al., 1999). According to this model, the frequency of an *Avr* gene in the pathogen population is counterbalanced by the frequency of the corresponding *R* gene in the plant population (Figure 2). Repeating cycles of epidemics of certain strains of a pathogen, resulting in rise and fall of the frequency of the corresponding *R* gene, cause maintenance of

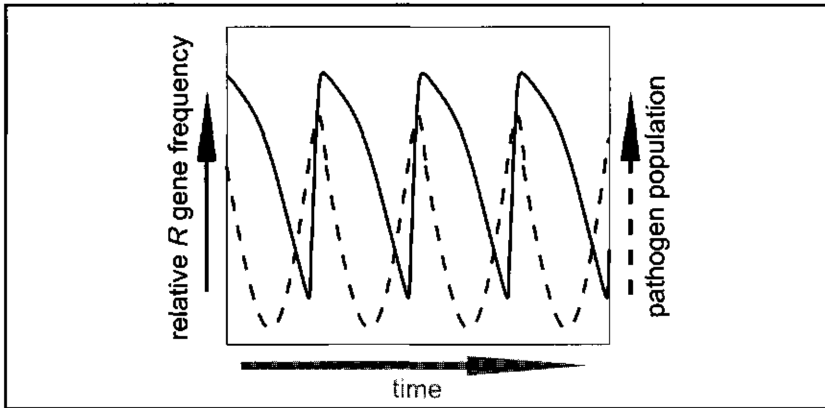

Figure 2. 'Trench-warfare' model for gene-for-gene interactions between plants and pathogens in nature. The frequency of occurrence of a particular *R* gene (solid line) fluctuates in time, following the density of the pathogen population carrying the matching *Avr* gene (dashed line). The figure is derived from Stahl et al. (1999).

certain specific gene-for-gene interactions over a long period of time. In addition, this model predicts that in nature, resistant plants co-exist with plants that lack the particular *R* gene.

In chapter 4 we have shown that recognitional specificity for AVR9, conferred by *Cf-9* and *9DC* genes, is present throughout the entire distribution range of *Lycopersicon pimpinellifolium*. It appears that in nature, AVR9-responsive plants co-exist with non-responsive plants. Previous studies on genetic markers in this population suggested that the species existed in a small centre of origin before it started to spread throughout its current distribution range. Thus, the dispersal of AVR9 recognition throughout the *L. pimpinellifolium* population suggests that this trait did not evolve recently. These data fully comply with the 'trench-warfare' model for gene-for-gene interactions in nature.

Loss of *Avr* genes results in a virulence penalty for the pathogen

It is obvious that carrying an *Avr* gene is not an advantage to the pathogen when it tries to infect a plant carrying the matching *R* gene. However, when attacking a susceptible plant, the Avr factor probably contributes to pathogen virulence, as is the case for many other factors that are produced by the pathogen during infection of the plant. For viruses, the virulence or pathogenicity function of *Avr* genes is often known. For example, the *Avr* gene matching the *Rx1* gene in potato encodes the coat protein of Potato Virus X (Bendahmane et al., 1995). Similarly, the coat protein of Turnip Crincle Virus acts as an elicitor on Arabidopsis carrying the *HRT* resistance gene (Zhao et al., 2000), whereas the helicase gene of Tobacco Mosaic Virus is recognised by tobacco carrying the *N* resistance gene (Abbink et al., 1998; Erickson et al., 1999). Plant-pathogenic bacteria lacking certain specific *Avr* genes often show reduced virulence. This has been observed for example for *avrBs2* (Kearney and Staskawicz, 1990); *avrRpm1* (Ritter et al., 1995); *avrRpt2* (Chen et al., 2000) and *avrPto* (Chang et al., 2000; Shan et al., 2000). For the barley leaf scald fungus, the *Avr* gene *Nip1* was found to contribute to pathogen fitness (Rohe et al., 1995). For many other *Avr* genes, like *Avr9* and *Avr4* of *C. fulvum*, no role in virulence has been

revealed yet. The role in pathogen fitness for an *Avr* gene is sometimes difficult to measure in laboratory experiments. For example, the *Ecp2* gene of *C. fulvum* appeared to be a virulence factor on mature tomato plants (Laugé et al., 1997), but not on seedlings (Marmeise et al., 1994). Similarly, a role of *avrPto* in pathogen fitness has not been observed in previous experiments (Ronald et al., 1992; Lorang et al., 1994), and the contribution of *avrRpt2* to pathogen fitness was only very small (Chen et al., 2000). To quantify the contribution of *Avr* genes to pathogen fitness, it would possibly be better to follow the frequency of a particular pathogen in the presence of other competing pathogens during a long time span with multiple generations.

The virulence penalty is the basis of 'trench-warfare'

During infection of a plant, pathogens use a diverse array of factors to obtain nutrients from the host and to suppress host defence responses. These factors may contribute to virulence at different levels. To cope with pathogens, plants generate *R* genes that have random specificity for pathogen factors. Some of these *R* genes confer recognition of factors that contribute significantly to virulence, whereas others confer recognition of less important factors. Factors that are recognised cause avirulence of the pathogen and the encoding genes are hence called *Avr* genes. To circumvent recognition, the pathogen can loose the *Avr* gene, but this will reduce pathogen fitness if the *Avr* gene has a role in virulence. Mutations in *Avr* genes that maintain the virulence function but abolish recognition are most valuable for the pathogen. Loss of recognition without a virulence penalty for the pathogen renders the matching *R* gene no longer functional, and this *R* gene will probably not be maintained in the natural plant population (Figure 3). Significantly, this selection will eventually result in the conservation of *R* genes that confer recognition that can not be circumvented by the pathogen unless a virulence penalty is taken. Similarly, only *Avr* genes that play a role in virulence will be maintained in the natural pathogen population, since loss of such an *Avr* gene will result in an impaired ability to infect plants and compete with other pathogens. Thus, only gene-for-gene couples where the *R* gene confers recognition of an *Avr* gene that contributes to virulence will continue to exist during 'trench-warfare'.

The 'guard' hypothesis

The key question now is: how can R proteins confer recognition of Avr factors that cannot be circumvented by the pathogen, without taking a virulence penalty? Although little is known about the virulence action of Avr factors, it is conceivable that they interact with virulence targets encoded by the host, in order to manipulate them in the advantage of the pathogen (Figure 4A). Virulence targets may consist of a single protein or multiple proteins and other factors. Manipulation of virulence targets might include a conformational change, (de)phosphorylation, recruitment or release of additional factors, etc. Recognition of the Avr factor based on its virulence function can be achieved by detection of the modification of a virulence target (Figure 4B). It should be stressed that in this model, the exact mechanism of R protein action remains open for speculation. Four possibilities are depicted in Fig. 1B, but in all cases, perception of the Avr factor involves its virulence target that is somehow 'guarded' by the matching R protein. Thus, R proteins might function as 'guards' that keep watch on an important virulence target ('treasure') of the Avr factor ('thief') (Figure 4C). Most important, the pathogen cannot

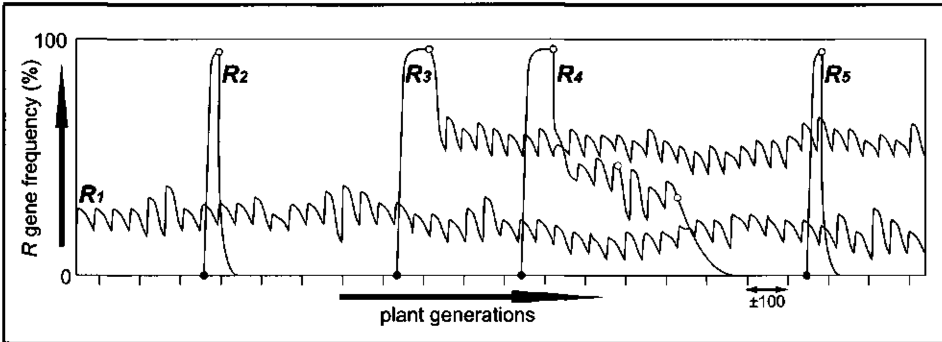

Figure 3. Simplified model of natural selection for *R* genes of which circumvention is associated with a fitness penalty for the pathogen. The frequency of various *R* genes ($R_{1\text{-}5}$) in the plant population over a long period of time is schematically represented. Circles indicate 'arms-race' events where new *R* genes are generated (black circle) or where pathogens circumvent recognition by a mutation in the matching *Avr* gene (white circle). Selection pressure after these events results in rise and fall, respectively, of the *R* gene frequency. Recognition of the *Avr* gene product by R_1 and R_3 gene products cannot be circumvented without a virulence penalty for the pathogen, resulting in maintenance of these genes in the population by 'trench-warfare' (fluctuating lines, Stahl et al., 1999). In contrast, *Avr* genes that match the R_2 and R_5 genes can be mutated without a virulence penalty for the pathogen. Consequently, the original *Avr* genes are not maintained in the pathogen population, and the matching *R* genes disappear from the plant population. Recognition by the R_4 gene product can only be circumvented after multiple mutations in the matching *Avr* gene.

circumvent this recognition, unless it avoids manipulating this particular virulence target, thereby taking a virulence penalty.

This 'guard' hypothesis complicates the simple biochemical 'ligand-receptor' interpretation of the gene-for-gene model, by predicting that a third component, the virulence target, is required for Avr-induced defence. A similar hypothesis was originally proposed for perception of AvrPto (Van der Biezen and Jones, 1998), and a literature search shows that many other gene-for-gene couples may comply with the 'guard' hypothesis (Table 2).

The *Pto* resistance gene of tomato confers recognition of AvrPto from *Pseudomonas syringae* pv. *tomato* (Martin et al., 1993; Ronald et al., 1992). AvrPto and Pto proteins physically interact (Scofield et al., 1996; Tang et al., 1996) and mutations that abolish the interaction, also abolish the induction of defence responses (Frederick et al.,1998; Chang et al., 2001; Shan et al., 2000). Although *Pto* is considered to be the *R* gene, its structure does not fit any *R* gene family since it contains no LRRs but encodes a serine/threonine kinase (Martin et al., 1993). Interestingly, in order to function, *Pto* depends on *Prf*, which encodes a resistance protein of the NBS-LRR class (Salmeron et al., 1996). It has been shown that *Prf* acts downstream of *Pto* (Rathjen et al., 1996). Therefore, it could well be that Prf is the R protein that guards the virulence target Pto and recognises modifications that take place in the Pto protein upon interaction with AvrPto (Van der Biezen and Jones, 1998). However, a physical interaction of Prf with Pto remains to be proven.

Table 2: Examples of gene-for-gene interactions for which the 'guard' hypothesis may apply

avirulence protein 'thief'	resistance protein 'guard'	virulence target 'treasure'
avrPto	Prf	Pto
avrPphB	RPS5	PBS1
CP	HRT	TIP
syringolide (avrD)	Rpg4*	P34
avrRpt2	RPS2	75kD*
AVR9	Cf-9	HABS*
AVR2	Cf-2	RCR3
AvrRpp5*	RPP5	AtRSH1

* corresponding gene not cloned yet

The *Rps5* resistance gene of Arabidopsis confers recognition of *avrPphB* from *Pseudomonas syringae* pv. *phaseolicola* (Warren et al., 1998). *Rps5* encodes a typical R protein of the NBS-LRR class, but its function depends on the presence of another plant gene, *PBS1* (Warren et al., 1999). Similar to *Pto*, *PBS1* encodes a serine/threonine kinase but it belongs to a distinct subfamily of protein kinases (Swiderski and Innes, 2001). Thus, it is unlikely that PBS1 and Pto fulfil the same function for the plant. A physical interaction between avrPphB and PBS1 or between PBS1 and RPS5 remains to be proven, but it has been speculated that RPS5 guards PBS1, which may act as virulence target for avrPphB (Swiderski and Innes, 2001).

As mentioned earlier, the *HRT* resistance gene of Arabidopsis confers recognition of the coat protein (CP) of Turnip Crinkle Virus (TCV) (Cooley et al., 2000; Zhao et al., 2000). The CP appears to interact with a plant protein, designated TIP (TCV-interacting protein) (Ren et al., 2000), which shares homology with the NAC-family of transcription factors, and can be considered as a virulence target of the CP. Mutations in the CP that abolish the interaction with TIP also abolish recognition by Arabidopsis carrying the *HRT* gene. It was therefore proposed that the HRT protein guards TIP and recognises modification by the CP (Ren et al., 2000).

The *Rpg4* resistance gene from soybean confers recognition of the *AvrD* gene of *Pseudomonas syringae* pv. *glycinea* (Kobayashi et al., 1990). The AvrD protein is not recognised itself, but mediates production of syringolides, which act as specific elicitors of defence responses in soybean carrying *Rpg4* (Keen et al., 1996). Syringolides bind to the vegetative storage protein P34, which has putative thiol-protease activity (Ji et al., 1998). This binding correlates with the recognition in soybean carrying *Rpg4* because derivatives of syringolides that show less affinity for P34, also have less elicitor activity (Ji et al., 1997). It can therefore be envisaged that the Rpg4 protein guards the P34 protein to recognise its modification by syringolides.

The *Rps2* resistance gene from Arabidopsis confers recognition of the *avrRpt2* gene product of *Pseudomonas syringae* pv. *maculicola* (Mindrinos et al., 1994; Bent et al., 1994; Innes et al., 1993). Interestingly, when expressed *in planta*, *avrRpt2* is able to complement the virulence penalty of bacterial strains that lack *avrRpt2* (Chen et al., 2000). Immunoprecipitations showed that the RPS2 and AvrRpt2 proteins form a complex that at least also includes a 75kD plant protein (Leister and Katagiri, 2000). The RPS2 and AvrRpt2 proteins also individually interact with probably the same 75kD protein. The gene encoding the 75kD protein has not been identified yet, and it has not been confirmed that it is

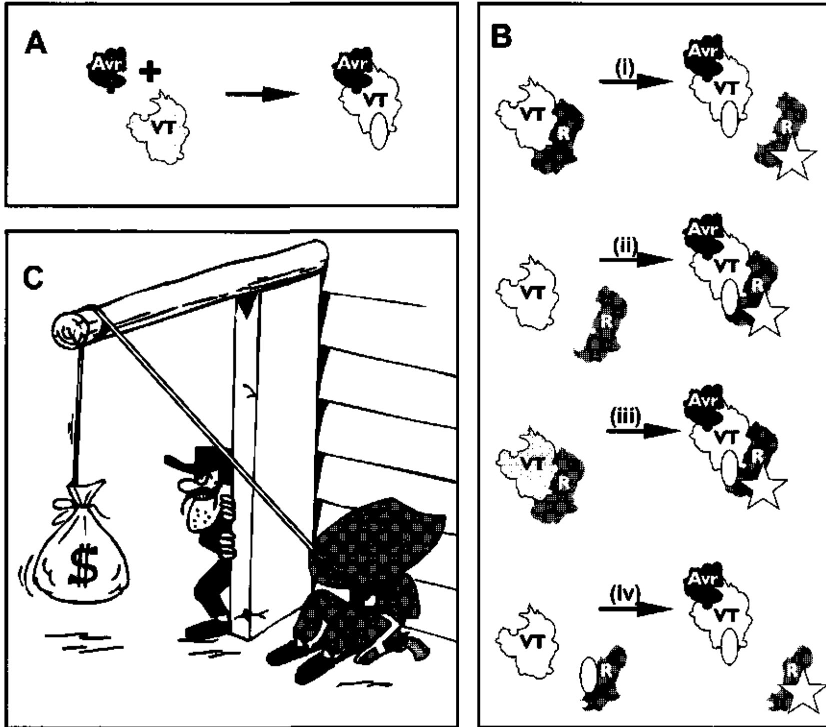

Figure 4. The 'guard' hypothesis
The 'guard' hypothesis implies that interaction of a pathogen-derived avirulence factor (Avr) with a virulence target (VT), encoded by the host, is required for recognition of the Avr by the resistance gene product (R).
A In susceptible plants, lacking the R protein, the Avr factor interacts with its VT, resulting in manipulation of the VT (oval). This manipulation may include conformational change, (de)phosphorylation, recruitment or release of additional factors, etc.
B In a resistant plant, the VT is 'guarded' by the R protein. Four examples are shown to illustrate possible mechanisms by which R proteins confer recognition and trigger a defence response (star). The R protein might: i, dissociate upon Avr binding to the VT; ii, bind to the VT upon Avr binding; iii, remain bound to the VT upon Avr binding; or iv, detect more distal Avr-induced modifications.
C The guard, the treasure and the thief, the major players in the 'guard' model.

required for *AvrRpt2* recognition by plants containing *Rps2*, but it is tempting to speculate that RPS2 guards this 75kD protein to monitor the formation of a complex with AvrRpt2.

As discussed in chapter 5, the Cf-9 resistance protein of tomato might guard the high-affinity binding site (HABS) for the corresponding AVR9 avirulence protein of *Cladosporium fulvum*. Evidence for physical interaction between Cf-9 and AVR9 was not found (Luderer et al., 2001). Mutations in AVR9 that affect the affinity for the HABS, affect the necrosis-inducing activity of these mutants in a similar way (Kooman-Gersmann et al., 1998). In addition, heterologous plant species only become AVR9-responsive upon transformation with *Cf-9*, when they contain the HABS (chapter 2; Hammond-Kosack et

al. 1998; Kooman-Gersmann et al., 1996). The widely spread occurrence of the HABS throughout the plant kingdom suggests that it fulfils a general role, other than AVR9 recognition, and that it may represent the pathogenicity target of AVR9, that is guarded by Cf-9 in resistant tomato plants.

The *Cf-2* resistance gene from tomato confers recognition of AVR2 secreted by *C. fulvum*. *Avr2* encodes a cysteine-rich protein (Luderer et al., manuscript in preparation), whereas *Cf-2* encodes a protein that is structurally very similar to Cf-4 and Cf-9 (Dixon et al., 1996). During mutant screens of plants carrying the *Cf-2* gene, a mutation designated *rcr3* (required for *Cladosporium* resistance-3) was identified that abolishes recognition of AVR2 (Dixon et al., 2000). Sequence similarity between Cf-2 and Cf-5 suggests that these R proteins use the same downstream signalling cascade. Although *Cf-5* is highly homologous to *Cf-2*, *Cf-5* function is not affected in an *rcr3* mutant background. As this suggests that RCR3 is specific for Cf-2, it has been proposed that Cf-2 guards RCR3 to recognise modifications induced upon interaction with AVR2 (Dixon et al., 2000).

The *RPP5* resistance gene of Arabidopsis confers resistance to *Peronospora parasitica* (Parker et al., 1997). The RPP5 protein interacts with AtRSH1, a RelA/Spot-like protein that transfers phosphate groups to the 3'-position of GDP and GTP, resulting in (p)pGpp, which may act as a second messenger in basal plant defence responses (Van der Biezen et al., 2000). This interaction appears to be specific for RPP5, because no interaction was found between AtRSH and RPP1, RPM1, RPS4 or the N protein. As AtRSH1 may act upstream of RPP5, it was proposed that RPP5 guards AtRSH1 to recognise a modification induced upon complexation with a yet unidentified avirulence protein of *Peronospora parasitica* (Van der Biezen et al., 2000).

In summary, for many gene-for-gene interactions there is support for the 'guard' hypothesis, although additional experiments are required to prove this concept. The 'guard' hypothesis may become a rule for gene-for-gene interactions, although there are already exceptions. For example, AvrPita of the rice blast fungus *Magnaporthe grisea* and the R protein Pi-ta of rice are known to directly interact (Jia et al., 2000). Important to mention is that *AvrPita* encodes a protein with putative protease activity which may fulfil the virulence function of this *Avr* gene (Orbach et al., 2000). Pi-ta, an NBS-LR protein (Table 1, chapter 1), appears to specifically interact with the proposed active site of AvrPita (Jia et al., 2000). Thus, also in this interaction, the virulence function of the Avr protein is recognised and recognition can only be circumvented if a virulence penalty is taken by the pathogen.

Dual specificity as additional advantage of the 'guard' hypothesis

In addition to providing a 'trap' that can be used for an indefinite period of time, the guarding function of R proteins may have an additional advantage. Different pathogens might use the same virulence target and guarding of such a virulence target may therefore give rise to multiple recognitional specificities mediated by a single R protein. There are indeed some indications that these multiple specificities are present in nature. The *Mi* resistance gene of tomato confers recognition of both aphids and nematodes, which are unlikely to produce the same Avr factor (Rossi et al., 1998; Vos et al., 1998). In addition,

the *RPM1* resistance gene of Arabidopsis mediates recognition of two unrelated avirulence proteins, *avrB* and *avrRpm1* of *Pseudomonas syringae* pv. *glycinea* and pv. *maculicola*, respectively (Grant et al., 1995).

Restricted taxonomic functionality of *R* genes

The striking similarity between *R* genes from different plant species suggests that most of these genes are functionally interchangeable. Thus, an *R* gene that confers resistance towards a pathogen with a broad host range may be useful to protect many crop plant species. For example, the *Bs2* gene from pepper is a candidate to confer broad range resistance towards bacterial spot in many different crop species (Tai et al., 1999). It might even be possible to use a certain wild plant species as a source of new *R* genes that confer recognition of proteins that are known to be essential pathogenicity factors of pathogens. Screening for an *R* gene that confers recognition of a virulence factor was successfully performed with the ECP2 protein of *C. fulvum* (Laugé et al. 1998). Future experiments may take this even one step further. With the knowledge where specificity resides in R proteins (chapter 3), and the availability of fast, transient expression systems (chapter 2), it might even be possible to artificially generate libraries of *R* gene mutants that differ in recognitional domains. Screening of such a library for the presence of a gene that confers recognition of a particular virulence factor may result in a novel, 'synthetic' gene that might confer resistance towards pathogens producing this virulence factor.

According to the 'guard' hypothesis, however, there is a major limitation to this strategy. The hypothesis implies that, in addition to the R protein, also the protein that is guarded by the R protein needs to be present in order to confer recognition of the matching Avr factor. The *R* gene will not be functional in plants that lack the virulence target of the corresponding Avr factor. During the past several years, many *R* genes have been reported to function in other plant species, but in most cases, their function was restricted to plants belonging to the same taxonomic group. For example, the *Bs2* gene from pepper only functions in solanaceous species but not in Arabidopsis, turnip, cucumber and broccoli (Tai et al., 1999). The same holds for *Cf-4* and *Cf-9*, which are functional in all solanaceous species tested, but not in Arabidopsis, flax, pea and lupine (chapter 2). Vice versa, the Arabidopsis *RPS2* gene is not functional in tomato (Dahlbeck and Staskawicz, unpublished). An exception to this so-called 'Restricted Taxonomic Functionality' (RTF) is provided by *Cf-4*, which is also active in lettuce (chapter 2). In summary, these examples illustrate that functionality of an *R* gene can not be guaranteed in a different plant species, perhaps because these species lack components that are required to form an active defence signalling complex, according to the 'guard' hypothesis.

Prospects for crop protection

The 'boom-and-bust' cycle illustrates that introduction of a resistant variety of a crop plant does often not result in durable protection against pathogens. In most cases, pathogens can easily circumvent recognition by the host plant. The virulence penalty that is taken by the pathogen by mutating an *Avr* gene can be small enough to yield a new strain of a pathogen that can still cause an epidemic in monocultures. In a natural situation, however, the pathogen will have to compete with other pathogens, which might be more successful. In this situation, the *Avr* gene is maintained in the pathogen

population. The frequency of a particular *R* gene in the plant population follows the frequency of occurrence of the matching *Avr* gene in the pathogen population. Thus, in nature, although complex gene-for-gene interactions evolve by the 'arms-race' between plant and pathogen, the individual players are maintained in their populations as a result of 'trench-warfare'. This explains how natural plant populations can survive under continuous attack by fast-evolving pathogens.

Understanding the role of gene-for-gene interactions in nature may help us to protect crop plants by introducing *R* genes in a way that simulates the natural situation. The 'mix-and-match' idea is such an approach (Pink and Puddephat, 1999). In this case, the crop consists of a mixture of plant lines that only differ in their *R* genes. The frequency of each *R* gene may be chosen, pending on the frequency of *Avr* genes that is detected in the pathogen population at the time of the previous harvest. It is likely that some plants in this 'multiline monoculture' become diseased, but epidemics are less likely to occur and pathogen pressure on the crop will be reduced as susceptible plants are surrounded by resistant plants. This strategy, in combination with additional strategies, could offer opportunities for integrated crop protection, to improve yield and quality in sustainable agriculture.

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Summary

To feed the increasing world population, agricultural production needs continuous improvement. Especially protection of crops from disastrous diseases is crucial. The interaction between the pathogenic fungus *Cladosporium fulvum* and its host, tomato, serves as a model system for plant-pathogen interactions. Some tomato plants carry resistance (*R*) genes that confer recognition of fungal strains carrying complementary avirulence (*Avr*) genes. A number of these *R* genes have been cloned, as well as their complementary *Avr* genes. The aim of the research described in this thesis was to examine how *R* gene products confer recognition of fungal strains carrying the matching *Avr* genes. Profound understanding of the molecular basis of this interaction might help us to improve the protection of other crop plants against economically important diseases.

Chapter 1 introduces the state of the art on interaction between *Cladosporium fulvum* and tomato at the time the research described in this thesis was initiated. *C. fulvum* is a specialised, biotrophic pathogen, causing tomato leaf mold. The fungus infects tomato leaves by entering stomata at the lower side of the leaf. The infection will proceed if no resistance *R* genes of the plant match any of the *Avr* genes of the fungus. However, the plant recognises the fungus when it carries an *R* gene that matches an *Avr* gene present in the fungus. This recognition results in the induction of plant defence responses, including a rapid death of cells surrounding the infection site, called the hypersensitive response (HR). Further fungal growth is prohibited by these defence responses. During its lifecycle on susceptible plants, *C. fulvum* is restricted to the extracellular space of the tomato leaves and secretes many proteins that potentially play a role in virulence. Also the elicitor proteins encoded by the *Avr9* and *Avr4* are secreted. Injection of these proteins is sufficient to trigger HR in tomato plants carrying *Cf-9* and *Cf-4* resistance genes, respectively. Both AVR9 and AVR4 are small, stable, cysteine-rich proteins. The complementary *Cf-9* and *Cf-4* genes encode highly similar, membrane-anchored, receptor-like proteins with extracytoplasmic leucine-rich repeats (LRRs) and a short cytoplasmic tail. Differences between Cf-9 and Cf-4 proteins are located in the N-terminal half, predominantly in amino acid residues at putative solvent-exposed positions of the LRRs, which is thought to form the 'recognition surface' of these proteins.

To examine the role of the various domains of Cf proteins in perception of AVR proteins of *C. fulvum* in more detail, a functional, transient expression system was developed for the *Cf-4* and *Cf-9* resistance genes (**chapter 2**). This expression system is based on infiltration of tobacco leaves with Agrobacterium strains that carry *Cf* genes on the T-DNA of binary plasmids (agroinfiltration). The AVR proteins are delivered either by injection, agroinfiltration, Potato Virus X-mediated expression or by using *Avr*-transgenic tobacco plants. This chapter also describes differences between *Avr9/Cf-9*- and *Avr4/Cf-4*-induced necrosis, which are mainly due to a difference in *Avr* gene activity upon expression in the plant. Finally, it is shown that the signal transduction pathway leading to HR is conserved in solanaceous plants, but likely not in

non-solanaceous plant species. An exception is the non-solanaceous plant lettuce, in which the *Avr4/Cf-4* gene pair is functional.
The agroinfiltration assay is an excellent expression system to study the effect of mutations in *Cf* genes. In **chapter 3**, agroinfiltration was used to determine specificity determinants in Cf proteins by exchanging domains between Cf-4 and Cf-9 and subsequently examining the effect of these mutations on specificity of perception of AVR proteins. Cf-4 differs from Cf-9 at 67 amino acid positions and also contains three deletions. Significantly, Cf-4 lacks two LRRs compared to Cf-9, which appears essential for Cf-4 function. The two additional LRRs in Cf-9 are required for Cf-9 function. Specificity determinants in Cf-4 reside not only in the LRR domain but also in the B-domain. In contrast, specificity determinants in Cf-9 reside entirely in the LRR domain and are likely scattered throughout this domain. The specificity determinants in the LRRs of Cf-4 cluster in a few adjacent LRRs and reside in only three amino acid residues at putative solvent-exposed positions. Thus, most of the 67 amino acids that vary between Cf-4 and Cf-9 appear not to be required for specificity, but probably serve as a source to generate new specificities.

To learn more about specificity determinants of Cf-9 proteins occurring in natural populations, we examined the molecular variation of Cf-9 in *Lycopersicon pimpinellifolium (Lp)*, from which the *Cf-9* locus has been introgressed into cultivated tomato (**chapter 4**). It appears that AVR9 recognition occurs frequently throughout the *Lp* population. In addition to *Cf-9*, a second gene, designated *9DC*, confers AVR9 recognition in *Lp*. Compared to *Cf-9*, *9DC* is more polymorphic, occurs more frequently and is more widely spread throughout the *Lp* population, suggesting that *9DC* is older than *Cf-9*. The second half of the *9DC* gene is nearly identical to the second half of *Cf-9*, whereas the first half is nearly identical to *Hcr9-9D*, a *Cf* homolog adjacent to *Cf-9* at the *Cf-9* locus. This suggests that *Cf-9* has evolved by intragenic recombination between *9DC* and another *Cf* homolog. The fact that 9DC and Cf-9 proteins both confer recognition of AVR9 but differ in 61 amino acid residues shows that Hcr9 proteins can be highly variable, without affecting their recognitional specificity.

After having examined their specificity determinants, we subsequently focused on the cellular location of Cf proteins. The presence of a dilysine motif in the G-domain of Cf-9 (KKRY) suggests that the protein resides in the endoplasmic reticulum (ER) instead of the plasma membrane (PM). Previously, two conflicting reports on the subcellular location of Cf-9 were published. One report showed that Cf-9 accumulates in the ER and is absent in the plasma membrane, whereas the other showed that Cf-9 resides in the plasma membrane. In **chapter 5** we have mutated the dilysine motif and show that the mutant Cf-9 protein remains functional in AVR9 recognition and mediation of HR. The data presented in this chapter, in combination with the two previous reports on Cf-9 localisation, can be explained by assuming that proteins that interact with Cf-9 mask the dilysine motif. This theory suggests that functional Cf-9 protein resides in small quantities in the plasma membrane, where it mediates recognition of the extracellular AVR9 protein as a component of a receptor complex.

AVR9 recognition in tomato plants carrying *Cf-9* most likely involves the high-affinity binding site (HABS) for AVR9 that was identified in plasma membranes. However, the HABS is not encoded by *Cf-9* because it is also present in tomato plants that lack *Cf-9* and in many other plant species. As it is likely that both the HABS and the Cf-9 protein reside in the plasma membrane and may be present in the same receptor complex, it is essential to isolate the HABS in order to get more insight in the molecular mechanism of AVR9 perception. In **chapter 6**, a procedure is described that allows solubilisation of the HABS without affecting its AVR9-binding activity. Of the 19 detergents that were tested, only octyl glucoside appeared to be suitable for solubilisation of the HABS. Removal of the detergent is crucial in this procedure, as it interferes with AVR9 binding. The described procedure may become an essential tool to study the AVR9 receptor complex at the biochemical level.

In the final chapter (**chapter 7**), the experimental data presented in the previous chapters are discussed. In addition to AVR9/Cf-9 there are many other examples of gene-for-gene interactions where no direct interaction was found between *R* and *Avr* gene products. In many cases, there are indications for the involvement of an additional host protein, which may represent the virulence target of the Avr protein. The prevalence of R proteins that 'guard' virulence targets can be explained by natural selection for *R* genes that are maintained in the plant population through 'trench-warfare', resulting in recognition events that cannot be circumvented by the pathogen without taking a virulence penalty. The 'guard' hypothesis significantly changes the focus of current research to the role of virulence targets of Avr proteins, and might explain absence of functionality of *R* genes in heterologous plant species, despite the fact that they belong to conserved gene families.

Samenvatting

Om de toenemende wereldbevolking te kunnen voeden, is een voortdurende productieverhoging in de landbouw noodzakelijk. Daarbij is de bescherming van gewassen tegen desastreuze ziekten cruciaal. De interactie tussen de schimmel *Cladosporium fulvum* en zijn gastheer tomaat dient als modelsysteem voor het onderzoek naar interacties tussen planten en hun ziekteverwekkers. Sommige tomatenplanten hebben resistentie (*R*) genen. Deze stellen de plant in staat een schimmel te herkennen wanneer deze een complementair avirulentie (*Avr*) gen bevat. Een aantal van deze *R* en *Avr* genenparen is gekloneerd. Doel van dit promotieonderzoek was uit te zoeken via welk mechanisme planten met een *R* gen in staat zijn een schimmel met het complementaire *Avr* gen te herkennen. Meer kennis op dit gebied zou ons kunnen helpen om de bescherming van belangrijke voedselgewassen tegen ziekten te verbeteren.

In **hoofdstuk 1** wordt de status van het onderzoek aan de interactie tussen *Cladosporium fulvum* en tomaat geïntroduceerd. Dit voor zover bekend was op het moment dat dit promotieonderzoek werd gestart. *C. fulvum* is een gespecialiseerde ziekteverwekker die de bladvlekkenziekte bij tomaat veroorzaakt. De schimmel infecteert de bladeren van tomatenplanten via de huidmondjes aan de onderkant van het blad. Het infectieproces zal doorgang vinden als er geen *R* genen in de plant aanwezig zijn, die complementair zijn aan de *Avr* genen van de schimmel. Wanneer dit wel het geval is, zal de plant de schimmel herkennen. Deze herkenning resulteert in het aanschakelen van een actieve afweerreactie, waarbij onder andere de cellen rondom de infectiehaard afsterven. Dit wordt ook wel de overgevoeligheidsreactie ('HR') genoemd. Door deze afweer is de schimmel niet in staat verder te groeien en is de plant resistent. Op vatbare planten groeit *C. fulvum* alleen in de ruimtes tussen de bladcellen en produceert daar verschillende eiwitten die mogelijk belangrijk zijn voor het infectieproces. Ook de elicitoreiwitten die door *Avr9* en *Avr4* worden gecodeerd, worden door de schimmel in de ruimtes tussen de bladcellen uitgescheiden. Injectie van deze elicitoreiwitten in het blad van de tomatenplant is voldoende om een overgevoeligheidsreactie te veroorzaken als de plant het resistentiegen *Cf-9,* respectievelijk *Cf-4* bevat. Zowel *Avr9* als *Avr4* coderen voor kleine, stabiele, cysteine-rijke eiwitten. De complementaire *Cf-9* en *Cf-4* genen coderen voor receptorachtige eiwitten die in de plasmamembraan verankerd zijn. Cf eiwitten hebben aan de buitenkant van de cel een aantal leucine-rijke repeats (LRRs) die kunnen binden aan andere extracellulaire eiwitten. Aan de binnenkant van de cel is slechts een klein 'staartje' van het Cf eiwit aanwezig. De verschillen tussen Cf-9 en Cf-4 eiwitten zijn niet groot. Deze verschillen bevinden zich in de N-terminale helft van de Cf eiwitten, vooral op plaatsen waar aminozuren aanwezig zijn die aan de buitenkant van de LRRs zitten en waarmee waarschijnlijk een specifiek herkenningsoppervlak gevormd wordt.

Om de rol van de verschillende domeinen in Cf eiwitten in de herkenning van AVRs van *C. fulvum* in meer detail te kunnen onderzoeken, is een functioneel expressiesysteem ontwikkeld voor *Cf-9* en *Cf-4* (**hoofdstuk 2**). Dit is gebaseerd op de infiltratie van

tabaksbladeren met Agrobacteriumstammen die *Cf* genen op het T-DNA van binaire plasmiden bevatten. Deze techniek wordt ook wel agroinfiltratie genoemd. De AVR eiwitten kunnen worden toegediend via injectie, agroinfiltratie, infectie met een virus die het *Avr* gen bevat, en door het gebruik van transgene planten die het *Avr* gen bevatten. Dit hoofdstuk beschrijft tevens de verschillen tussen de overgevoeligheidsreacties geïnduceerd door *Avr9/Cf-9* en *Avr4/Cf-4*, welke vooral het gevolg zijn van een verschil in activiteit van de *Avr* genen tijdens de expressie in de plant. Tenslotte wordt getoond dat de signaaltransductieroute die leidt tot HR, geconserveerd is in de nachtschadenfamilie, maar waarschijnlijk niet in planten die niet tot deze familie behoren. Een uitzondering hierop vormt sla, behorende tot de composietenfamilie, waarin het *Avr4/Cf-4* genpaar functioneel is.

Agroinfiltratie is een uitstekend middel om het effect van mutaties in Cf eiwitten te bestuderen. In **hoofdstuk 3** is agroinfiltratie gebruikt om te bepalen welke domeinen en aminozuren de specificiteit van Cf eiwitten bepalen. Dit is gedaan door domeinen tussen Cf-4 en Cf-9 uit te wisselen en vervolgens te kijken naar het effect hiervan op de herkenning van AVR4 en AVR9 eiwitten. Cf-4 verschilt van Cf-9 op 67 aminozuurposities en bevat drie deleties. Cf-4 mist twee LRRs in vergelijking met Cf-9 en deze blijken belangrijk te zijn voor Cf-4 functie. De twee extra LRRs in Cf-9 zijn belangrijk voor Cf-9 functie. Specificiteit in Cf-4 zit niet alleen in het LRR domein, maar ook in het B-domein. Dit in tegenstelling tot Cf-9, waar de specificiteit zich enkel in de LRRs bevindt en waarschijnlijk verspreid is over dit gehele domein. De specificiteit in het LRR domein van Cf-4 bevindt zich in naast elkaar gelegen LRRs en bestaat uit slechts drie aminozuren op posities die zich waarschijnlijk aan de buitenzijde van het eiwit bevinden. Het blijkt dat de meeste van de 67 aminozuren die verschillen tussen Cf-4 en Cf-9 niet vereist zijn voor specificiteit, maar waarschijnlijk dienen als een bron voor het genereren van nieuwe specificiteiten.

Om meer te weten te komen over de aspecten in Cf-9 eiwitten die specificiteit bepalen, hebben we naar de variatie van Cf-9 in de natuurlijke populatie van *Lycopersicon pimpinellifolium* (*Lp*) gekeken (**hoofdstuk 4**). Het *Cf-9* locus dat we kennen van de gecultiveerde tomaat (*L. esculentum*), is vanuit deze soort ingekruist. Het blijkt dat herkenning van AVR9 veel voorkomt in de gehele *Lp* populatie. Naast *Cf-9* is een tweede gen ontdekt, genaamd *9DC*, dat de herkenning van AVR9 in *Lp* veroorzaakt. In vergelijking met *Cf-9* komt *9DC* meer voor in de populatie, is meer verspreid over de regio's waar *Lp* voorkomt en bevatten de *9DC* gensequenties meer mutaties. Dit suggereert dat *9DC* ouder is dan *Cf-9*. Het tweede deel van het *9DC* gen is vrijwel identiek aan de tweede helft van *Cf-9*, terwijl de eerste helft vrijwel identiek is aan *Hcr9-9D*, een *Cf* genhomoloog welke naast *Cf-9* in het *Cf-9* locus ligt. Dit suggereert dat *Cf-9* is ontstaan via een intragene recombinatie tussen *9DC* en een ander *Cf* genhomoloog. Het feit dat 9DC en Cf-9 eiwitten beide AVR9 herkenning veroorzaken, maar toch op 61 aminozuur posities van elkaar verschillen, laat zien dat Cf eiwitten sterk kunnen variëren zonder effect op hun specificiteit.

Nadat de specificiteit in Cf eiwitten onderzocht was, hebben we ons gericht op de cellulaire locatie van Cf eiwitten. De aanwezigheid van een dilysine motief in het C-terminale domein van Cf-9 (KKRY) suggereert dat het eiwit zich in het endoplasmatisch reticulum (ER) bevindt, in plaats van in de plasmamembraan (PM). Over de lokalisatie van Cf-9 zijn

recentelijk twee publicaties verschenen waarvan de resultaten duidelijk tegenstrijdig waren. Eén publicatie rapporteerde dat Cf-9 in het ER accumuleert en afwezig is in de PM, terwijl de andere publicatie liet zien dat Cf-9 in de PM zit. In **hoofdstuk 5** hebben we het dilysine motief gemuteerd, waardoor het Cf-9 eiwit niet meer in het ER kan accumuleren en we tonen aan dat de Cf-9 mutant nog steeds in staat is om herkenning van AVR9 te veroorzaken, met de inductie van de HR als gevolg. De resultaten uit dit hoofdstuk en die uit de twee eerder verschenen publicaties, kunnen worden verklaard met het model dat eiwitten die mogelijk binden aan Cf-9, in staat zijn het dilysine signaal af te schermen. Deze theorie suggereert dat functioneel Cf-9 zich in kleine hoeveelheden in de PM bevindt, alwaar het, als onderdeel van een receptor complex, een rol speelt bij de herkenning van het extracellulaire AVR9 eiwit.

De herkenning van AVR9 door tomatenplanten die *Cf-9* bevatten vereist waarschijnlijk de aanwezigheid van de hoge-affiniteits bindingsplaats ('HABS') voor AVR9, die in plasmamembranen geïdentificeerd is. Echter, de HABS wordt niet gecodeerd door *Cf-9* zelf, omdat deze bindingsplaats ook aanwezig is in tomatenplanten zonder *Cf-9* en ook in vele andere plantensoorten. Omdat het vermoeden bestaat dat zowel de HABS als Cf-9 eiwit een receptorcomplex vormen in de plasmamembraan, is het interessant om de HABS te zuiveren om meer inzicht te krijgen in het moleculaire mechanisme van AVR9 herkenning. In **hoofdstuk 6** is een procedure beschreven voor het in oplossing brengen van de HABS uit membraanpreparaten, zonder verandering van zijn AVR9-bindende activiteit. Van de 19 zepen die zijn getest, bleek alleen octyl glucoside geschikt voor het in oplossing brengen van de HABS. Het verwijderen van het zeep is cruciaal in deze studies omdat het interfereert in de binding met AVR9. De beschreven procedure kan een essentieel gereedschap vormen voor de studie naar het AVR9 receptorcomplex op biochemisch niveau.

In het laatste hoofdstuk (**hoofdstuk** 7) worden de experimentele data van de voorgaande hoofdstukken bediscussieerd. Naast AVR9/Cf-9 bestaan er nog veel andere voorbeelden van gen-om-gen interacties waarbij geen directe interactie tussen avirulentie- en resistentie-eiwitten is gevonden. In veel gevallen blijkt dat de specifieke herkenning van de ziekteverwekker extra factoren van de gastheer vereist. Vaak kunnen deze factoren het virulentiedoelwit van het Avr eiwit zijn. De meeste resistentie-eiwitten lijken deze virulentiedoelwitten te 'bewaken'. Deze trend kan verklaard worden door een natuurlijke selectie op resistentiegenen die, via een 'loopgraven oorlog' met de ziekteverwekkers, in de plantenpopulatie behouden blijven, omdat zij een herkenning veroorzaken die niet vermeden kan worden zonder een vermindering van virulentie van de ziekteverwekker. Dit model van resistentie-eiwitten die virulentie doelwitten 'bewaken' zal de focus van huidig onderzoek verplaatsen naar de virulentie doelwitten van Avr eiwitten. Bovendien kan het de beperkte taxonomische uitwisselbaarheid van resistentiegenen verklaren, ondanks het feit dat ze behoren tot geconserveerde genfamilies.

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Curriculum Vitae

Renier Adrianus Leonardus van der Hoorn werd op 27 juni 1971 in Leiden geboren. Op de potplantenkwekerij van zijn ouders te Roelofarendsveen ontstond zijn grote interesse in planten. In 1987 slaagde hij voor zijn MAVO examen en drie jaar later voor zijn VWO examen, beide met hoge cijfers. In 1990 begon hij met de studie scheikunde aan de Universiteit van Leiden, waar hij zich specialiseerde in moleculaire biologie van planten, onder andere door colleges in Wageningen te volgen. Hij onderbrak zijn studie gedurende een half jaar toen hij deel uitmaakte van de universitaire ElCid-commissie, die de jaarlijkse introductieweek voor eerstejaars studenten organiseert. Voor zijn afstudeervakken heeft hij onderzoek gedaan aan systemische resistentie in tabak (sectie plantenvirussen, prof. John Bol, Universiteit Leiden) en 'silencing' van bloemkleurgenen in Petunia (sectie moleculaire genetica, prof. Jos Mol, Vrije Universiteit, Amsterdam). Na het behalen van zijn bul in 1996, met een 8.4 als gemiddeld cijfer, kreeg hij de unieke kans om als onderzoeker in opleiding in dienst te treden bij de vakgroep Fytopathologie, Universiteit Wageningen. Dit onderzoek was een door NWO gefinancierd project en resulteerde in dit proefschrift. Sinds 1 januari 2001 heeft hij een aanstelling als onderzoeker bij dezelfde groep, om het functioneren van Cf eiwitten verder te onderzoeken.

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