Molecular evolution of *Cladosporium fulvum* disease resistance genes in wild tomato

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General introduction and outline

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General introduction and outline of the thesis

General introduction

Plant crops provide a primary source of human food source and cattle feed. In the face of a fast growing world population, it is vital to sustain and improve crop yields. A major challenge is to increase crop yields by reducing losses due to plant diseases. Chemical control is an effective means to control plant pathogens, but can have detrimental effects on the environment. Preferably, resistance of plants to pathogens is achieved by exploiting natural resistance mechanisms. Transfer of resistance genes (*R* genes) of wild plant species into crop plants is an effective and environmentally safe way of crop protection. However, the resistance provided by these *R* genes has often been broken by newly emerging pathogen strains (Agrios, 1997). Knowledge of *R* genes in natural plant populations, and understanding of the forces that drive *R* gene evolution may lead to better methods to protect agriculturally important crop plants against their pathogens. Potentially durable *R* genes may be identified, and also a better use of known *R* genes in crop plants may be achieved (Jones, 2001).

Resistance of plants against pathogens

Resistance of an entire plant species against a certain pathogen is usually called nonhost resistance, and is the most common type of disease resistance exhibited by plants (Heath, 2000). However, plants from species that are susceptible to a certain pathogen may still exhibit two types of resistance. Horizontal, polygenic resistance provides a basal level of protection to all races of a pathogen (Agrios, 1997). Although this type of resistance does not render plants completely immune to pathogens, it is durable and slows down the spread of the disease and the development of epidemics in the field. In contrast, vertical resistance is mediated by monogenic *R* genes and provides absolute protection against pathogens (Agrios, 1997). However, this type of resistance is often broken by the pathogen when it adapts as a result of the selection pressure imposed by the *R* gene. By studies on the flax-*Melampsora lini* pathosystem, Flor genetically explained the breakdown of *R* genes by the gene-for-gene model (Flor, 1942, 1946, 1971). This model states that for each *R* gene in the plant, the pathogen carries a corresponding avirulence gene (*Avr* gene). Unless a plant carries an *R* gene and the invading pathogen strain carries the corresponding *Avr* gene, the pathogen will successfully attack the plant, which will consequently become diseased.

To date, many *R* genes have been isolated (reviewed in Hammond-Kosack and Parker, 2003; Hulbert et al., 2001; Martin et al., 2003; Nimchuk et al., 2003; Takken and Joosten, 2000). Some *R* genes encode proteins with no structural homology or with a specific function. For instance, the first cloned *R* gene, *Hm1* from maize, encodes an HC-toxin reductase that detoxifies the HC-toxin of the maize pathogen *Cochliobolus carbonum* and thereby confers resistance (Johal and Briggs, 1992). However, most *R* genes encode proteins

belonging to five major structural classes. One class is presented by the intracellular serine/threonine Pto kinase from tomato. Most R proteins, however, contain a nucleotide-binding site (NBS) and carry leucine-rich repeats (LRRs). At the N-terminus, these NBS-LRR proteins carry either a region with similarity to the N-terminus of the Toll and Interleukin 1 receptor (TIR-NBS-LRR proteins), or a leucine-zipper (LZ) or other coiled-coil motif (CC-NBS-LRR proteins). All NBS-LRR proteins are predicted to be intracellular. The fourth class comprises R proteins like the tomato Cf proteins, which contain a transmembrane (TM) domain and extracellular LRRs. Xa21 from rice shares the extracellular LRRs and the TM domain with the Cf-like proteins, but in addition carries an intracellular serine/threonine kinase domain. Most *R* genes are part of a gene-for-gene type of relationship that involves the specific recognition of a pathogen elicitor, after which a defence response is mounted that often includes a hypersensitive response (HR) (Hammond-Kosack and Jones, 1996; Nimchuk et al., 2003). As a consequence, pathogen growth is restricted, and the plant remains resistant. Although the function of *R* genes is obvious, for many pathogen *Avr* genes an intrinsic function is yet to be assigned (Van 't Slot and Knogge, 2002). Avr proteins not only trigger defence responses in resistant plants, but likely contribute to pathogen virulence in susceptible plants as well (Brown, 2003; Van der Hoorn et al., 2002).

Resistance gene evolution in natural pathosystems

In the ongoing battle between plants and their pathogens in their natural habitats, plant pathogens are under selection pressure to circumvent or break resistance imposed by their host plants, whereas plants require functional *R* genes to effectively counteract their pathogens. Two major hypotheses exist that describe the dynamics of *R* gene evolution (Figure 1). The birth-and-death model, or arms race model, assumes that an *R* gene emerges in a plant population (birth), and will go extinct in the plant population when the resistance is broken (death) (Michelmore and Meyers, 1998). New *R* genes should be continuously generated to ensure adequate protection against pathogens. In the second model, the trench warfare model, *R* genes are maintained in the plant population under balancing selection over long periods (Stahl et al., 1999). The frequency of an *R* gene in the plant population depends on the selective advantage it offers to the plant (reviewed in Bergelson et al., 2001; Holub, 2001). The matching *Avr* gene is hypothesised to contribute to the fitness of the pathogen, and is therefore subject to a negative selection pressure imposed by the *R* gene in the host plant. As a result, the *Avr* gene frequency will fluctuate in time (Brown, 2003; Van der Hoorn et al., 2002). Therefore, the selective advantage the *R* gene provides to the host plant also changes, and as a consequence the *R* gene frequency in the plant population also fluctuates in time (Figure 1). Recent studies suggest that *R* genes are maintained in plant populations over long periods of time and may be subject to balancing selection (Mauricio et al., 2003; Riely and Martin, 2001; Rose et al., 2004; Stahl et al., 1999; Tian et al., 2002). However, the *Rps4* gene from *Arabidopsis thaliana* may have evolved in compliance with the arms race model (Bergelson et al., 2001). Therefore, both the arms race and the trench warfare model may

reflect the versatile strategies that plants employ to counteract their pathogens during evolution. Convergent evolution of *R* genes against the AvrB elicitor from *Pseudomonas syringae* in soybean and *Arabidopsis thaliana* was shown recently (Ashfield et al., 2004). Thus, *R* genes in different plant species may be conserved and ancient, or may have evolved independently to acquire the same function.

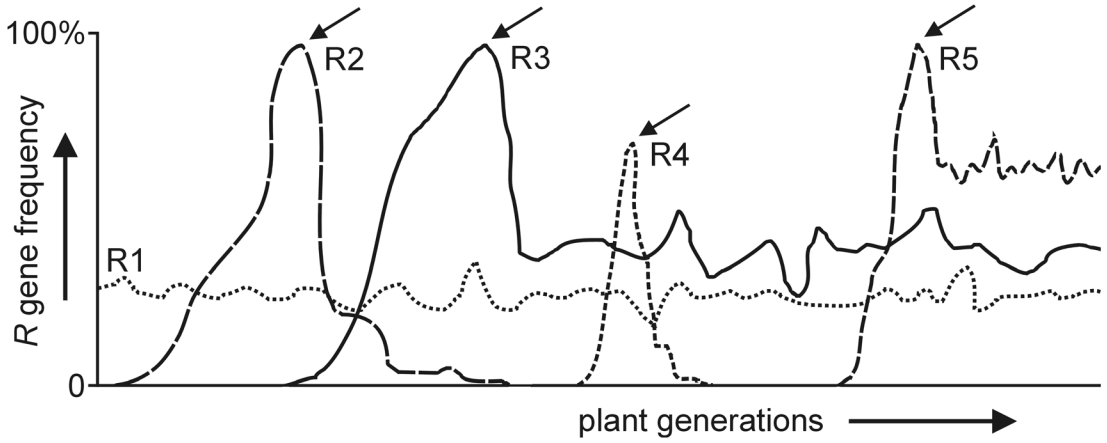


Figure 1. Schematic representation of two models of evolutionary *R* gene dynamics.
The frequency of the *R* genes in the plant population is plotted against time, expressed as an increasing number of plant generations. Arrows indicate events in time *R* genes have been overcome by the pathogen. *R2* and *R4* represent *R* genes that follow the *birth-and-death* or *arms race* model. These genes are born, provide a selective advantage to the plant and will increase in frequency in the plant population. Consequently, selection pressure is imposed upon the pathogen to overcome the novel *R* gene. As soon as the pathogen has circumvented the *R* gene, the *R* gene has become obsolete and will disappear from the plant population. *R1*, *R3* and *R5* represent *R* genes that follow the *trench warfare* model, and are subject to *balancing selection*. These *R* genes persist in the plant population despite the presence of virulent pathogen strains, as the pathogen suffers a fitness cost to circumvent the *R* gene. The relatively stable frequencies of *R1*, *R3* and *R5* reflect the fitness cost imposed on the pathogen to overcome these genes. The higher the fitness cost imposed on the pathogen, the higher the frequency of the *R* gene in the plant population will be.

In a single plant population, many different *R* genes may be present (Cattan-Toupance et al., 1998; Laugé et al., 2000). Although individual plants carry only a limited repertoire of *R* genes, the protection provided by the pool of *R* genes in the population and the geographical distribution of plants within a population prevent the emergence of 'super-races' of pathogens (Thrall and Burdon, 2002, 2003). In contrast, a limited repertoire of *R* genes is present in crop monocultures, which may lead to virulent pathogen strains that can devastate crops in subsequent growing seasons. The complex *R* gene structure in natural plant populations can be mimicked successfully in agricultural settings by deploying mixtures of cultivars or lines with different *R* genes (so-called multilines) (Jones, 2001; Wolfe, 1985; Zhu et al., 2000).

The tomato-*Cladosporium fulvum* pathosystem

The *Cladosporium fulvum*-tomato pathosystem is an ideal model system for studying the interaction between biotrophic pathogenic fungi and plants, and complies with the gene-for-gene model (Joosten and De Wit, 1999; Rivas and Thomas, 2002). *C. fulvum* is a biotrophic leaf pathogen, of which tomato (*Lycopersicon*) species are the only reported hosts. Upon germination, *C. fulvum* runner hyphae enter the tomato leaf via stomata on the lower side of the leaf. The fungus does not from specialized feeding structures, but grows in the apoplastic space of susceptible tomato leaves in close contact with the mesophyll cells where it obtains nutrients from the plant (Figure 2A). After approximately 10 days, substomatal stroma are formed and aerial mycelium with conidiophores emerges through the stomata that produces the asexual conidia (Figure 2B). In the incompatible interaction, the fungal hyphae are recognised upon entry, which results in HR of cells surrounding the infection site, thereby restricting further fungal growth (Figure 2C). As a result, the resistant leaf remains undiseased (Figure 2D).

During infection, *C. fulvum* secretes numerous small proteins into the apoplast. These small proteins can act as elicitors of resistance in tomato plants that carry a corresponding *C. fulvum* resistance (*Cf*) gene (Laugé et al., 2000). Eight different elicitor genes have been isolated thus far, and all encode proteins with an even number of cysteines, which likely contribute to protein stability through the formation of disulfide bridges (reviewed in Joosten and De Wit, 1999). These elicitors are divided into Avr and Ecp (Extracellular proteins) proteins. Some *C. fulvum* isolates do not produce all wild type Avr elicitor proteins that allow recognition of and resistance to *C. fulvum* in tomato lines that carry the matching *Cf* genes. Therefore, the *Avr* genes encode race-specific elicitors. The *Avr2*, *Avr4*, *Avr4E* and *Avr9* genes have all been isolated, and *C. fulvum* was shown to avoid recognition in *Cf-2*, *Cf-4, Cf-4E* and *Cf-9* tomato lines via the production of unstable elicitor proteins, frame-shift or non-sense mutations in the *Avr* genes, deletion of the *Avr* gene or insertion of a transposon in an *Avr* open reading frame (Joosten et al., 1994; Luderer et al., 2002; Van Kan et al., 1991; Westerink et al., 2003). In contrast to the race-specific *Avr* genes, *Ecp* genes are secreted by all known *C. fulvum* isolates. The Ecp1-Ecp5 proteins have all been purified, and all corresponding genes, except *Ecp3*, have been isolated (Van den Ackerveken et al., 1993a; Laugé et al., 2000. So far, little sequence variation in the *Ecp* genes has been found compared to the *Avr* genes (De Kock et al., personal communication). This may reflect the fact that the corresponding *Cf-Ecp* genes have not been deployed in large-scale breeding and production programs, or suggests that the *Ecp* genes play an important role in pathogenicity, as was observed for *Ecp1* and *Ecp2* (Laugé *et al*., 1997). Consistent with the gene-for-gene model, recognition of each of the *C. fulvum* Avr and Ecp elicitor proteins has been found in different wild tomato plants and tomato breeding lines (Laugé et al., 1998b, 2000).

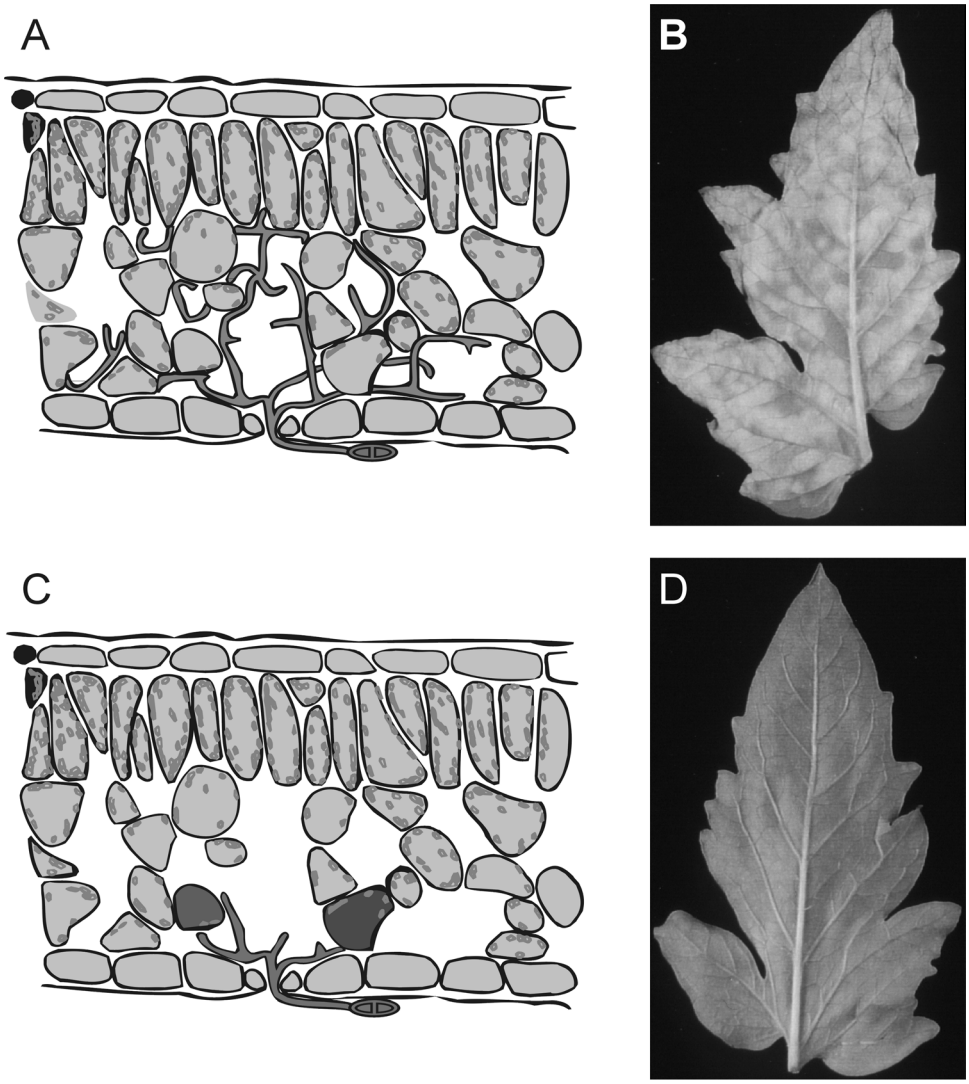


Figure 2. The compatible and incompatible interactions between tomato and *Cladosporium fulvum*.
A. Schematic representation of a cross section of a susceptible tomato leaf after inoculation with a virulent *C. fulvum* strain. A runner hyphae has entered the leaf mesophyll through an open stoma, after which *C. fulvum* colonises the extracellular leaf space.
B. Lower side of a susceptible tomato leaf, two weeks after inoculation with a virulent *C. fulvum* strain (compatible interaction). The fungus sporulates abundantly.
C. Schematic representation of a cross section of a resistant tomato leaf after inoculation with an avirulent *C. fulvum* strain. Upon entering the leaf through an open stoma, *C. fulvum* is recognised, which results in a hypersensitive response (indicated as dark cells) that restricts further fungal growth.
D. Lower side of a resistant tomato leaf, two weeks after inoculation with an avirulent *C. fulvum* strain (incompatible interaction). No signs of fungal infection are visible.

The Avr4 and Avr9 elicitors of *Cladosporium fulvum*

The two best-studied *C. fulvum* elicitors are Avr4 and Avr9 (Figure 3A). Both proteins were isolated using various biochemical separation techniques, and by analysis of the HR-inducing activity of protein fractions by injections into leaflets of *Cf-4* and *Cf-9* tomato plants (Joosten et al., 1994; Scholtens-Toma and De Wit, 1988). Avr4 encodes a mature peptide of 86 to 88 amino acids, depending on the degree of N- and C-terminal processing (Joosten et al., 1997). Avr4 was shown to bind chitin oligomers, and may protect the *C. fulvum* cell wall against plant chitinases during infection of plants or against fungal hyperparasites that secrete chitinases (Van der Burg et al., 2003). *C. fulvum* races that have overcome *Cf-4*-mediated resistance produce unstable or non-functional Avr proteins (Joosten et al., 1997). Most unstable Avr4 proteins contain cysteine to tyrosine mutations involved in two disulfide bonds that are conserved in an invertebrate chitin-binding domain, yet these unstable proteins retained their chitin-binding ability (Van den Burg et al., 2003). Therefore, these mutations do not seem to affect the intrinsic function of Avr4, and allow *C. fulvum* to circumvent *Cf-4*-mediated resistance.

The mature Avr9 protein contains only 28 amino acids (Scholtens-Toma and De Wit, 1988). Avr9 secreted by an Avr9-overexpressing *C. fulvum* strain was shown to consist of 32, 33 or 34 amino acids (Van den Ackerveken et al., 1993b). This suggests that the mature Avr9 protein of 28 amino acid is the result of further processing by plant proteases. The 33 amino acid Avr9 protein was purified and shown to contain a cysteine knot, which consists of a ring formed by two disulfide bridges and the interconnecting backbone through which the third disulfide bridge penetrates (Vervoort et al., 1997). This structural motif is found in several small proteins, such as proteinase inhibitors, ion channel blockers and growth factors. Furthermore, the Avr9 protein is structurally very homologous to carboxy peptidase inhibitor, but seems not to function as such (Vervoort et al., 1997). The Avr9 protein has also been chemically synthesized and folded *in vitro* (Van den Hooven et al., 1999). No intrinsic function has been reported for Avr9 yet, however, the homology with other proteins suggests that it may interfere with natural regulatory processes in the tomato leaf. A high-affinity binding site for Avr9 is present in plasma membranes of all tested Solanaceous species, but not in other plant species (Kooman-Gersmann et al., 1998). Moreover, no direct interaction between Cf-9 and Avr9 was found (Luderer et al., 2001). This high-affinity binding site may therefore represent a putative virulence target of Avr9. *Avr9* gene replacement did not affect the pathogenic fitness of *C. fulvum* in greenhouse infection assays (Marmeisse et al., 1993). *C. fulvum* races isolated from cultivated tomato that circumvent *Cf-9*-mediated resistance were shown to have lost the *Avr9* gene (Van Kan et al., 1991).

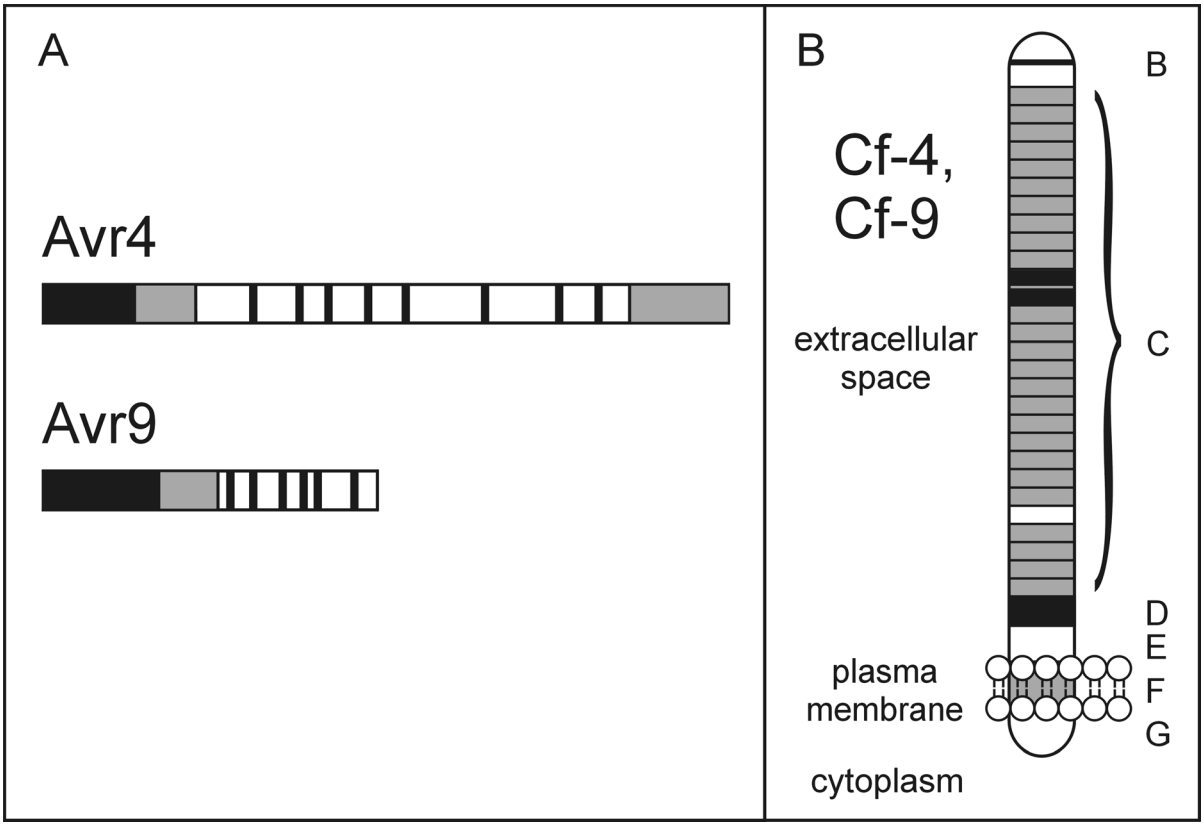


Figure 3. Schematic representation of the *Cladosporium fulvum* elicitor proteins Avr4 and Avr9 and the *C. fulvum* resistance proteins Cf-4 and Cf-9 from tomato.

A. Schematic representation of the *C. fulvum* Avr4 and Avr9 elicitor proteins. The mature elicitors are depicted in white; cysteine residues in the mature elicitors are indicated by black vertical bars. Black boxes indicate signal peptides for extracellular targeting, and grey regions are processed *in planta* by endogenous plant proteases.

B. Schematic representation of the mature *C. fulvum* resistance proteins Cf-4 and Cf-9 from tomato. Cf-4 and Cf-9 comprise several functional domains (Jones et al., 1994; Jones and Jones, 1997), indicated as domain B-H. The signal peptide for extracellular targeting (domain A) is not present in the mature protein. Domains B-E are located in the extracellular space; domain F is located in the plasma membrane; domain G is cytoplasmic. Domain B (white) is cysteine-rich, the black bar indicates a deletion of 10 amino acids in Cf-4 relative to Cf-9; domain C of Cf-9 comprises 27 leucine-rich repeats (LRRs; grey) and a loop-out (white) between LRRs 23 and 24. LRRs 11 and 12 (indicated in black) are not present in Cf-4, which carries 25 LRRs; domain D (black) has no distinct features; domain E (grey) is acidic; domain F (grey) is a transmembrane domain; domain G is a basic cytoplasmic tail.

Cladosporium fulvum resistance genes in tomato

Eight wild tomato species, as well as the wild ancestor of *L. esculentum* (cultivated tomato), are found in diverse habitats on the west coast of South America from southern Ecuador to northern Chile and on the Galapagos Islands (Warnock, 1988, 1991). These wild tomato species are a rich source of *C. fulvum* resistance genes, of which some have been introgressed into cultivated tomato. Near-isogenic lines of the susceptible tomato cultivar MoneyMaker that contain the *Cf-2*, *Cf-4*, *Cf-5* or *Cf-9* resistance genes have been generated (Boukema, 1981, Tigchelaar, 1984). These four *Cf*-genes have all been isolated, as well as the *Cf-4E* gene from MoneyMaker-Cf4 (Dixon et al., 1996, 1998; Jones et al., 1994; Takken et al., 1999; Thomas et al., 1997). *Cf-4*, *Cf-4E* and *Cf-9* are all members of the *Hcr9* (Homologues of *C. fulvum* resistance gene *Cf-9*) gene family, whereas *Cf-2* and *Cf-5* are members of the *Hcr2* gene family (Homologues of *C. fulvum* resistance gene *Cf-2*).

Cf-9 was the first isolated *Cf* gene (Jones et al., 1994), and *Cf-4* is highly related to *Cf-9* (Thomas et al., 1997). The isolation of the *Avr4* and *Avr9* elicitor genes from *C. fulvum* allowed detailed studies of *Cf-4*- and *Cf-9*-based elicitor perception and resistance (Joosten and De Wit, 1999; Rivas and Thomas, 2002). Therefore, *Cf-4* and *Cf-9* are the best-studied *Cf* genes. The Cf-4 and Cf-9 proteins contain several structural domains, including 25 and 27 extracellular leucine-rich repeats (LRRs), respectively, and a transmembrane domain with a short cytoplasmic tail (Figure 3B) (Jones et al., 1994; Jones and Jones, 1997). Several features in Cf-4 and Cf-9 have been identified that are essential for their function (Van der Hoorn et al., 2001a; Wulff, et al., 2001). However, despite considerable efforts, the mechanism of elicitor perception and activation of defence responses mediated by *Cf* genes is yet poorly understood.

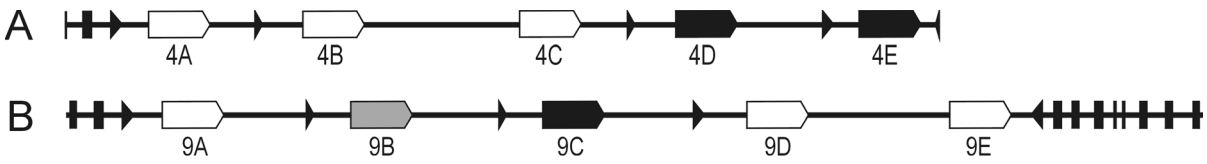


Figure 4. Schematic representation of the *Cf-4* and *Cf-9 Cladosporium fulvum* resistance gene clusters.
Arrowed boxes represent *Cf* gene homologues and indicate their transcriptional polarity. Black homologues represent functional *Cf* genes that confer resistance to *C. fulvum*; the grey homologue represents a *Cf* gene that confers partial resistance to *C. fulvum*; white homologues have no known function in resistance to *C. fulvum*. Black arrows and boxes represent exon fragments of the *LipoxygenaseC* gene, some of which have been co-duplicated with *Cf* gene homologues (Parniske et al., 1997).
A. The *Cf-4* cluster from the wild tomato species *L. hirsutum*, which harbours the *C. fulvum* resistance genes *Cf-4* (*4D*) and *Cf-4E* (*4E*) (Parniske et al., 1997; Takken et al., 1999; Thomas et al., 1997).
B. The *Cf-9* cluster from the wild tomato species *L. pimpinellifolium*, which harbours the *C. fulvum* resistance gene *Cf-9* (*9C*), and the partial resistance gene *Hcr9-9B* (*9B*) (Jones et al., 1994; Parniske et al. 1997; Laugé et al., 1998a).

Genetic mechanisms involved in plant disease resistance gene evolution

R genes evolve by several genetic mechanisms. Single genes may evolve by accumulation of point mutations and incidental sequence exchange between different alleles. However, *R* genes are frequently located in clusters of tandemly repeated homologues. Usually one or a few of these homologues have a known function in disease resistance (Figure 4) (reviewed in Hulbert et al., 2001; Leister, 2004; Michelmore and Meyers, 1998; Takken and Joosten, 2000).

The clustering facilitates mispairing of conserved *R* gene homologues during meiosis, which allows sequence exchange by unequal recombination or gene-conversion. A high rate of sequence exchange will lead to sequence homogenisation of homologues, and ultimately to loss of variation. To prevent sequence homogenisation of homologues and retain sequence exchange, the rate of sequence exchange between *R* gene homologues should be regulated by the intergenic regions (IRs) between the homologues (Parniske et al., 1997). These IRs are rapidly diverging sequences due to lack of selection pressure, and may therefore limit mispairing of the homologues. This provides a means to reduce the sequence exchange rate, allowing both generation of novel diversity and conservation of existing *R* genes. New combinations of *R* gene homologues within a single *R* gene cluster may be formed by intergenic recombination, whereas intragenic recombination and gene-conversion may yield novel *R* genes. Intragenic unequal recombination may also increase or decrease the number of repeat regions, such as LRR coding regions, within *R* gene homologues. Point mutations may further contribute to the divergence of individual *R* genes. Finally, translocation of *R* gene homologues may lead to the generation of novel *R* gene clusters (Figure 5).

Hallmark papers by Parniske and co-workers describe studies of the *Hcr9* gene family and have provided novel insights in the evolution of *R* gene clusters (Parniske et al., 1997, 1999; Parniske and Jones, 1999). *Hcr9*s are found at least at five different loci on the short arm of chromosome 1 of tomato (*Northern Lights*, *Milky Way*, *Aurora*, *Orion* and *Southern Cross*) (Parniske et al., 1997, 1999; Parniske and Jones, 1999; Haanstra et al., 1999, 2000a). Several *Hcr9* clusters have been isolated thus far, including the *Cf-4* and *Cf-9* clusters (Figure 4) (Parniske et al., 1997; Parniske and Jones, 1999). The *Hcr9* gene family not only comprises *Hcr9*s that mediate resistance to *C. fulvum*, but also *Hcr9*s with no known biological function (Jones et al., 1994; Parniske et al., 1997; Parniske and Jones, 1999; Thomas et al., 1997). The *Hcr9*s were shown to be composed of 'patchwork' sequences, suggesting extensive sequence exchange between homologues. Translocation, duplication, inter- and intragenic unequal recombination, gene-conversion, point mutation and insertion and deletion likely have all contributed to *Hcr9* sequence diversity (Figure 5). *Hcr9*s that do not confer resistance to *C. fulvum* may therefore represent a reservoir of variation that may be used in the generation of novel *Cf* genes.

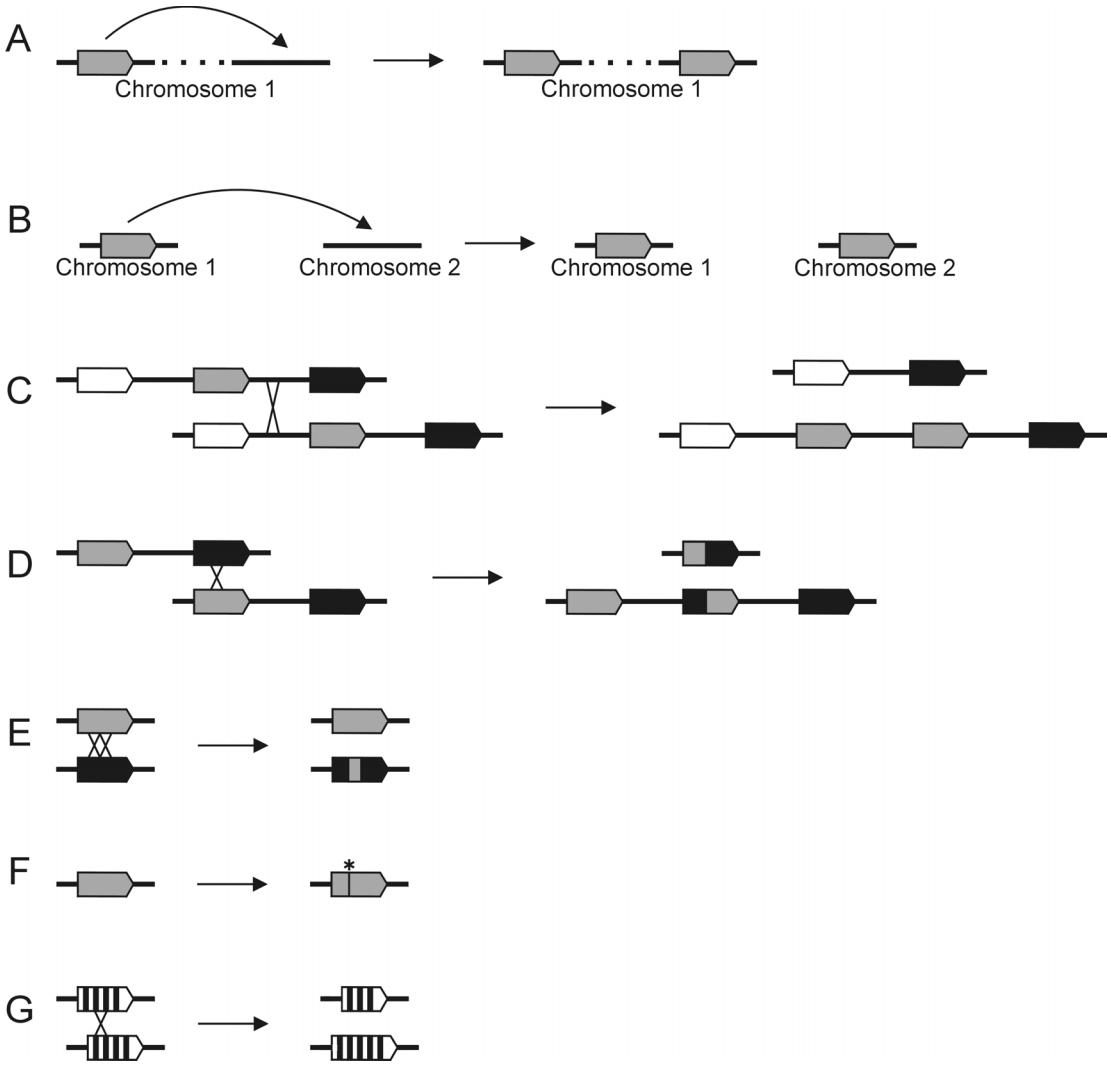


Figure 5. Genetic mechanisms involved in the generation of novel *R* gene variation.
On the left, the initial situations are shown; horizontal arrows point towards the resulting situations on the right. Arrowed boxes represent *R* gene homologues and different colours (white, grey and black) indicate different *R* gene homologue sequences; diagonal crossing lines indicate (unequal) recombination or gene conversion points. All recombination and gene conversion events are shown in a homozygous background.
A. *Translocation* of an ancestral *R* gene homologue to a region on the same chromosome.
B. *Translocation* of an ancestral *R* gene homologue to a region on a different chromosome.
C. *Intergenic recombination* leads to novel combinations of *R* gene homologues, leads to *duplication* of *R* gene homologues and can also alter the number of *R* gene homologues in a cluster.
D. *Intragenic recombination* leads to novel chimeric *R* gene homologues and can also alter the number of *R* gene homologues in a cluster.
E. *Gene conversion* leads to sequence exchange within *R* gene homologues and creates novel chimeric *R* gene homologues.
F. *Point mutation* may generate novel variation within an *R* gene homologue. The point mutation is indicated by an asterisk and a vertical black line within the *R* gene homologue.

Outline of the thesis

The evolution of the tomato *Hcr9* gene family, which comprises several *C. fulvum* disease resistance genes, has been well documented by isolation and comparison of several *Hcr9* clusters from different species and chromosomal positions. However, variation within *Hcr9*s that mediate responsiveness to the same elicitor has not been described, and is the main subject of the studies presented in this thesis. A comprehensive study of the evolution of *C. fulvum* disease resistance genes that mediate recognition of Avr4 and Avr9 in wild tomato populations is described.

The *Cf-9* gene originates from *L. pimpinellifolium* and provides resistance to races of *C. fulvum* that secrete Avr9. The *Cf-9* cluster comprises five homologues, of which *Hcr9-9C* is the *Cf-9* gene. In a study on molecular variation of *Cf-9* in the *L. pimpinellifolium* population, the *9DC* gene was discovered, which also confers Avr9 recognition (**chapter 2**). Avr9 recognition occurs throughout the *L. pimpinellifolium* population, and is likely only mediated by the *9DC* and *Cf-9* genes. *9DC* occurs at a higher frequency in the *L. pimpinellifolium* population than *Cf-9* and is more widely spread, which indicated that *9DC* might predate *Cf-9*. The sequences of *9DC* and *Cf-9* further suggested that *Cf-9* evolved from *9DC* by intragenic recombination between *9DC* and another *Hcr9*. *9DC* has the same activity and specificity as *Cf-9*, although it differs by 61 amino acids, which shows that natural Hcr9 proteins with the same recognitional specificity may vary significantly.

The close evolution of the *Cf-9* and *9DC* genes was studied in more detail by the isolation of the complete *9DC* cluster from *L. pimpinellifolium* LA1301 (**chapter 3**), from which *9DC* was isolated. A phage library was screened for phages containing *9DC*, and *9DC*-containing clones were assembled into a contig and sequenced. The *9DC* cluster contains three *9DC* genes (*9DC1*, *9DC2* and *9DC3*), orthologues of the *Hcr9-9A* and *Hcr9-9E* genes from the *Cf-9* cluster, as well as three *Hcr9* fragments. Comparison of the *Cf-9* and *9DC* gene clusters provided strong indications that *Cf-9* is ancestral to *9DC*, in contrast to what was suggested in chapter 2. Multiple unequal inter- and intragenic recombination events have resulted in the three *9DC* genes. All three *9DC* genes confer Avr9 recognition in agroinfiltration overexpression assays in tobacco. However, only *9DC2* is active upon agroinfiltration under control of its native promoter, and is therefore most likely the main determinant of Avr9 recognition in *L. pimpinellifolium* LA1301.

The low variation in *Cf-9* and *9DC* in the *L. pimpinellifolium* population suggested that *Cf* genes might be highly conserved due to selection. However, the complex evolution of the *Hcr9* gene family could also allow convergent evolution of *Cf* genes with the same specificity. To address the question how *Hcr9*s that recognise the same *C. fulvum* elicitor have evolved, the variation in functional homologues of *Cf-4* and *Cf-9* was studied in wild tomato species (**chapter 4**). Avr4- and Avr9-responsive plants were identified throughout the *Lycopersicon* genus. *Hcr9*s from different responding *Lycopersicon* plants were amplified by

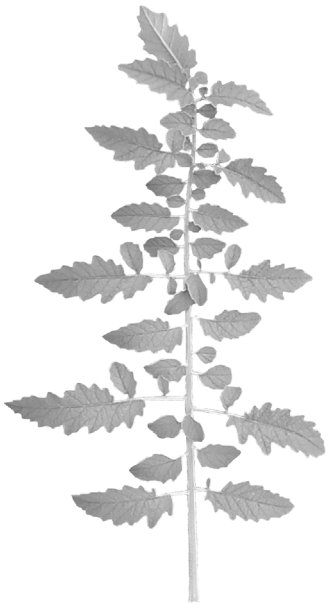
PCR and single *Hcr9*s were tested for conferring Avr4 or Avr9 responsiveness by agroinfiltration overexpression assays in tobacco. The isolation of highly conserved functional homologues of *Cf-4* and *Cf-9* from diverged tomato species strongly suggested that these genes are ancient and predate *Lycopersicon* speciation. Moreover, these results suggest that *C. fulvum* is an ancient pathogen of the *Lycopersicon* genus and has imposed selection pressure on *Lycopersicon* plants, resulting in the maintenance of functional homologues of *Cf-4* and *Cf-9* in different wild tomato species.

In our screen for functional homologues of *Cf-4* and *Cf-9*, we also identified two *Hcr9*s from *L. peruvianum*, which induced an elicitor-independent HR upon transient overexpression by agroinfiltration in several tobacco species, and named them auto-activators of HR (**chapter 5**). Additional auto-activators were generated by *in vitro* shuffling of *Hcr9* sequences. The auto-activators exhibited different HR-inducing activites in five selected tobacco species, and were shown to function in the same signalling pathway as *Cf-9*. Several models to explain the elicitor-independence of the auto-activators and their potential use in unravelling the mechanisms of *Cf* gene-mediated resistance are discussed.

The general discussion (**chapter 6**) gives an overview on the current status of the research on *Cf*-like disease resistance genes in plants. The genetic mechanisms and dynamics of evolution of *Cf*-like disease resistance genes are described, as well as the variation and evolution of *Cf* genes in plant populations. The pathosystems from which *Cf*-like disease resistance genes have been isolated are also briefly described. The current status of the research on signal perception and signal transduction mediated by *Cf*-like genes is discussed, with emphasis on the extensively studied tomato *Cf* genes.

Chapter 2

Intragenic recombination generated two distinct *Cf* genes that mediate Avr9 recognition in the natural population of *Lycopersicon pimpinellifolium*



Renier A.L. Van der Hoorn*, Marco Kruijt*, Ronelle Roth, Bas F. Brandwagt,
Matthieu H.A.J. Joosten and Pierre J.G.M. De Wit (2001)
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*: These authors have contributed equally.

Intragenic recombination generated two distinct *Cf* genes that mediate Avr9 recognition in the natural population of *Lycopersicon pimpinellifolium*

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 homologues (*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.

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 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; Riely 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 (Van der Hoorn et al., 2001a). In Cf-9, specificity is likely scattered throughout the LRR domain (Van der Hoorn et al., 2001a; Wulff et al., 2001).

The *Cf-9* gene is the first described member of a gene family, called *Hcr9*s (Homologues 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 homologues, named *Hcr9-9A* to *-9E*. The *Cf-9* locus has been introgressed into cultivated tomato (*Lycopersicon esculentum*) from its wild relative *L. pimpinellifolium* (*Lp*) (Tigchelaar, 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.

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 characterise 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 (Van der Hoorn et al., 2001a). 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).

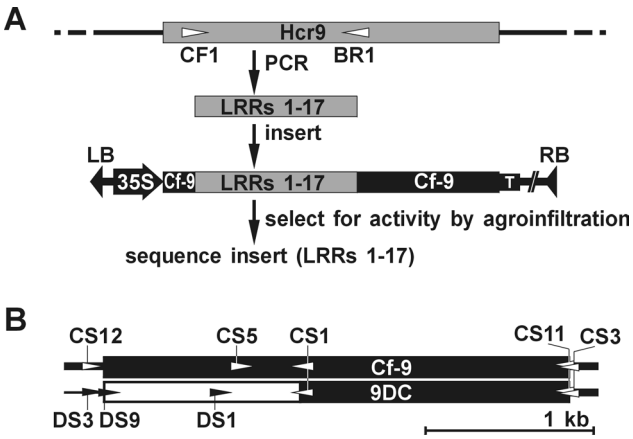

Figure 1. Selection procedure for chimeric *Hcr9* constructs that confer Avr9 recognition and position of the various specific primers.

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.

B. Positions of specific primers in and flanking the *Cf-9* and *9DC* ORFs. Triangles indicate the annealing positions and directions of the primers relative to the ORF of *Cf-9* and *9DC*.

Transient co-expression of the chimeric *Cf-9* genes with *Avr9* in tobacco by agroinfiltration (Van der Hoorn et al., 2000) 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 (0.4 kb, encoding LRRs 12-17) was identical to *Cf-9* (*Hcr9-9C*), whereas the 5'-part (0.8 kb, 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*.

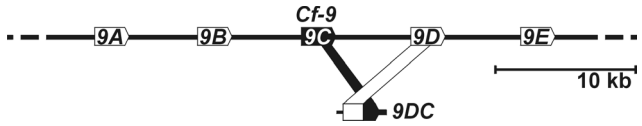

Figure 2. Organisation of the *Hcr9*s at the *Cf-9* locus and their homology with *9DC*.
The five *Hcr9*s (*9A* to *9E*) present at the *Cf-9* locus are indicated (top) as well as the area of homology of *Hcr9-9C* and *Hcr9-9D* with *9DC* (bottom). Arrows indicate ORFs with transcriptional direction. 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.

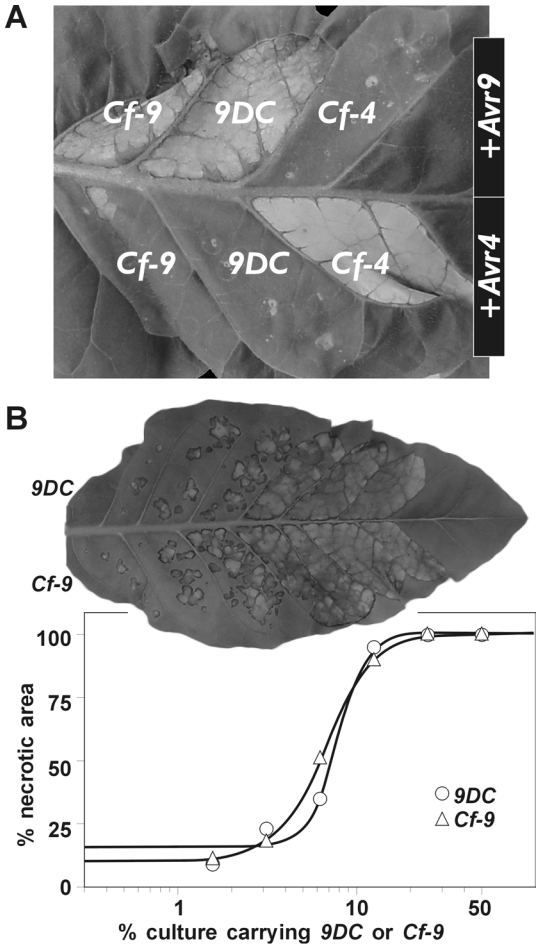

Figure 3. Comparative transient expression studies of *9DC* and *Cf-9*.

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. The 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.

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 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-recognising plants are present throughout the geographical distribution range of *Lp* (data not shown). To calculate

the 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 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.

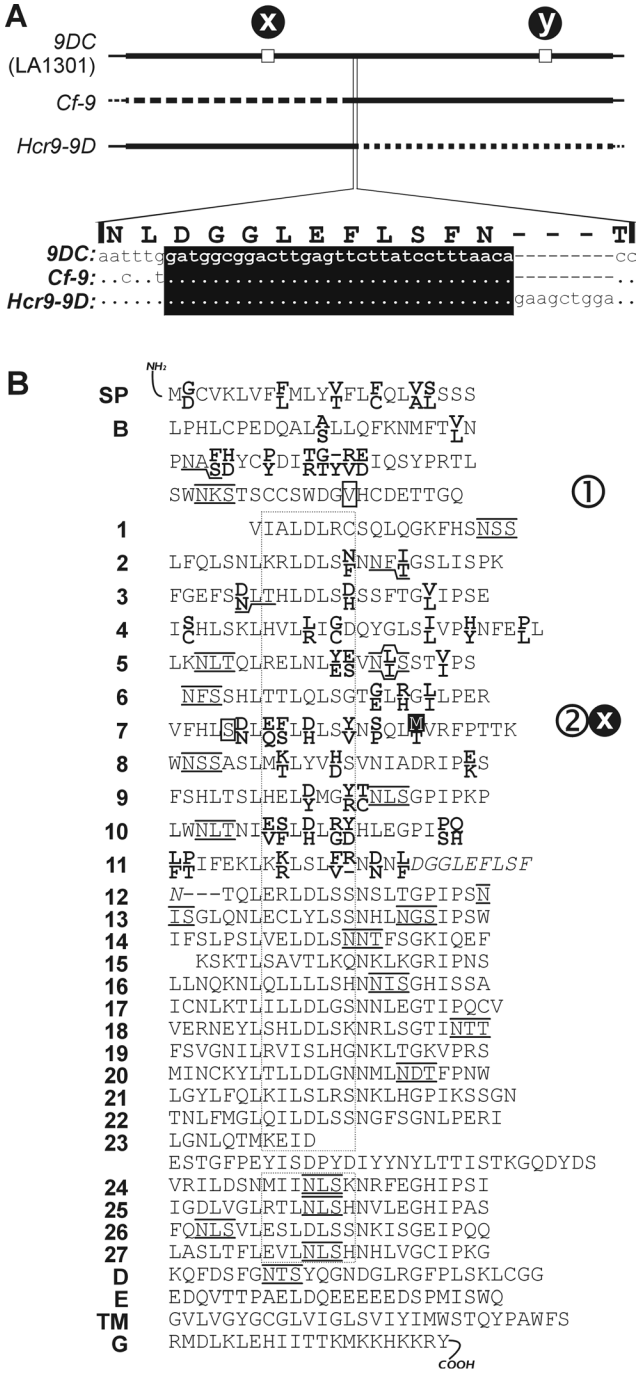

Figure 4. Comparison of nucleotide and protein sequences of *9DC, Cf-9* and *Hcr9-9D*.

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 dotted 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.

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 (Van der Hoorn et al., 2001a). 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 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; see also chapter 3). 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 (Van der Hoorn et al., 2001a; 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).

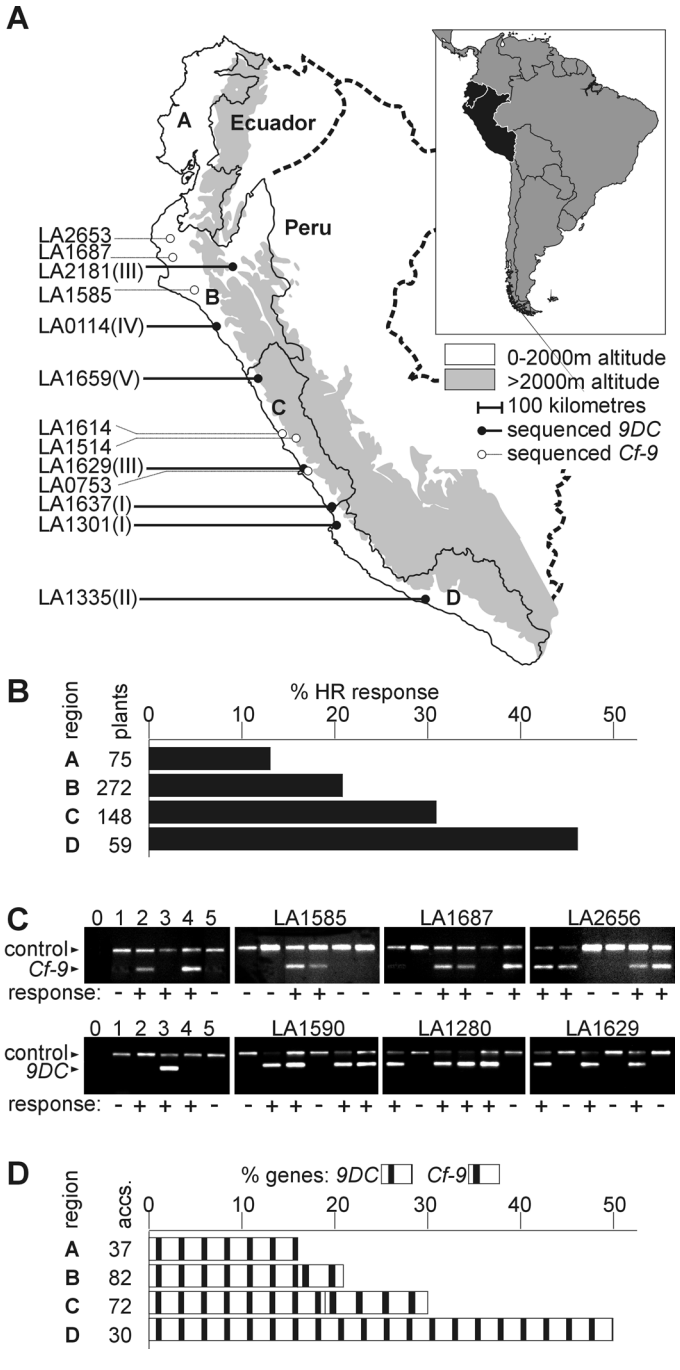

Figure 5. Frequency of Avr9-recognition and occurrence of *9DC* and *Cf-9* in the *Lp* population.

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.

Intragenic recombination between *R* gene homologues

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 homologue that was not functional in Avr2 recognition (Dixon et al., 1998). Also, intragenic recombination between the functional *RPP8* gene and its adjacent homologue *RPP8A* probably resulted in an inactive *rpp8* homologue (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*.

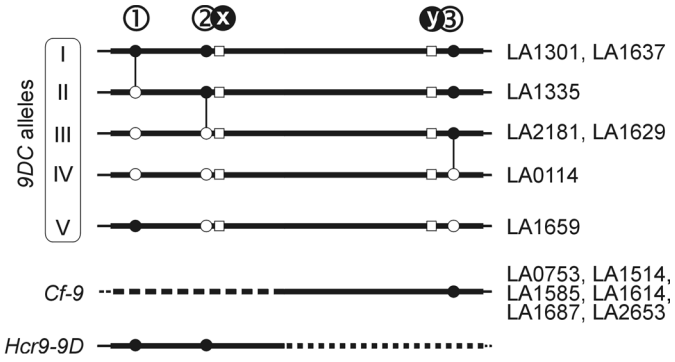

Figure 6. Polymorphism among *9DC* alleles and their relation with *Cf-9* and *Hcr9-9D*.
Accessions from which *9DC* or *Cf-9* have been sequenced are indicated on the right. Solid lines represent DNA sequences of the *9DC* alleles. Thick lines indicate ORFs. Open squares at positions x and y indicate 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.

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, favouring incidence of *C. fulvum*. Coastal temperatures in southern Peru range from 15-22 degrees compared to 18-25 degrees in Ecuador (Warnock, 1991). A more moderate temperature is known to favour the occurrence of tomato leaf mould (Small, 1930).

Avr9 recognition in the *Lp* population complies with the 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 centre 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-recognising 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

Plant material

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

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* (Stratagene, La Jolla, CA) or with the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Restriction enzymes, T4 ligase, and *Escherichia coli* 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:ccccctgcagtcactaatatcttttcttgtgc, 2606; CS12: tcttctctatcaacataacaag, -44; DS1: gagagctcaacctttacgaa, 587; DS3: ctatgtgaggtagctagtag, -124; DS9: tttttccatgggttgtgtaaaacttgtg, -7.

Construction of chimeric *Hcr9*s

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 (Van der Hoorn et al., 2001a) using *Cla*I and *Bam*HI restriction sites, thereby constructing chimeric *Hcr9*s (Figure 1A).

Detection of *9DC* and *Cf-9* in *Lp* plants

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).

Isolation and sequencing of *9DC* and *Cf-9* alleles

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. The sequence of the *9DC* gene of *L. pimpinellifolium* LA1301 has been deposited with GenBank under accession number AF401036.

Functionality testing of *9DC*

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 (Van der Hoorn et al., 2000), using *Nco*I and *Pst*I restriction sites. The 35S-*9DC*-T cassette was subsequently inserted into the binary plasmid pMOG800 (Honée et al., 1998), using *Xba*I and *Kpn*I restriction sites. Agroinfiltration of tobacco plants (*Nicotiana tabacum* cv. Petite Havana SR1) was performed as described (Van der Hoorn et al., 2000). To compare the activity of *9DC* with *Cf-9*, dilution series of *Agrobacterium* cultures were infiltrated and necrotic responses were quantified as described (Van der Hoorn et al., 2001a).

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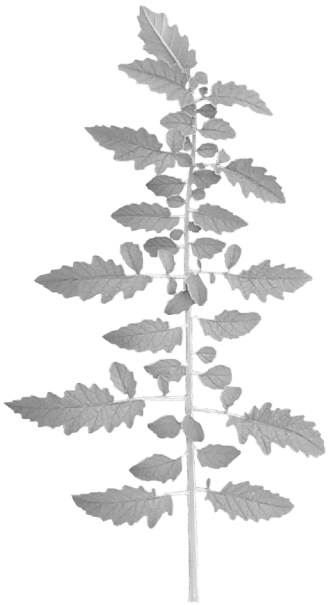
Chapter 3

Rearrangements in the *Cf-9* disease resistance gene cluster of wild tomato have resulted in three genes that mediate Avr9 responsiveness



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Rearrangements in the *Cf-9* disease resistance gene cluster of wild tomato have resulted in three genes that mediate Avr9 responsiveness

Abstract

***Cf*-resistance genes in tomato confer resistance to the fungal leaf pathogen *Cladosporium fulvum*. Both the well-characterised resistance gene *Cf-9* and the related *9DC* gene confer resistance to strains of *C. fulvum* that secrete the Avr9 protein and originate from the wild tomato species *Lycopersicon pimpinellifolium*. We show that *9DC* and *Cf-9* are allelic, and we have isolated and sequenced the complete *9DC* cluster of *L. pimpinellifolium* LA1301. This *9DC* cluster harbours five full-length *Cf* homologues, including orthologues of the most distal homologues of the *Cf-9* cluster, and three central *9DC* genes. Two *9DC* genes have an identical coding sequence (*9DC1* and *9DC2*), whereas *9DC3* differs at its 3' terminus. From a detailed comparison of the *9DC* and *Cf-9* clusters, we conclude that the *Cf-9* and *Hcr9-9D* genes from the *Cf-9* cluster are ancestral to the first *9DC* gene, and that the three *9DC* genes were generated by subsequent intra- and intergenic unequal recombination events. Thus, the *9DC* cluster has undergone substantial rearrangements in the central region, but not at the ends. Using transient transformation assays, we show that all three *9DC* genes confer Avr9 responsiveness, but that *9DC2* is likely the main determinant of Avr9 recognition in LA1301.**

Plants are continuously challenged by a diverse array of pathogens. Resistant plants carry resistance (*R*) genes that enable them to recognise pathogen strains that carry matching avirulence (*Avr*) genes, a phenomenon called the gene-for-gene relationship (Flor, 1946). In response to pathogen pressure, sophisticated surveillance systems have evolved to maintain and generate new *R* genes in plants (Hulbert et al., 2001; Michelmore and Meyers 1998). *R* genes often occur in clusters, and extensive sequence exchange between homologues can occur through unequal recombination. This leads to novel sequence combinations and possibly to novel *R* genes. Although many *R* genes and *R* gene clusters have been isolated, the evolution of *R* gene clusters in natural populations is still poorly understood.

One of the best-studied pathosystems that follows the gene-for-gene relationship is the tomato-*Cladosporium fulvum* interaction (Joosten and De Wit, 1999; Rivas and Thomas 2002). *C. fulvum* is a fungal biotrophic leaf pathogen of tomato (*Lycopersicon*) species. Resistance of tomato cultivars to *C. fulvum* has been introduced from wild tomato germplasm. The tomato resistance gene *Cf-9* that originates from *L. pimpinellifolium* (*Lp*) confers resistance to strains of *C. fulvum* that secrete the Avr9 protein (Jones et al., 1994). In

resistant *Cf-9* plants, a hypersensitive response (HR) is mounted at the infection site upon Avr9 recognition, thereby restricting the growth of the fungus. A high proportion of *Lp* accessions collected from their natural habitat is able to recognise Avr9 (Laugé et al., 2000; chapter 2), which suggests that the ability to recognise Avr9 may be beneficial to wild tomato plants.

Cf-9 is a member of the *Hcr9* (Homologues of *Cladosporium fulvum* resistance gene *Cf-9*) gene family (Parniske et al., 1997). *Hcr9*s encode proteins with a stretch of extracellular leucine-rich repeats, a transmembrane domain and a short cytoplasmic tail that lacks an obvious signalling signature (Joosten and De Wit, 1999; Rivas and Thomas, 2002). Tomato plants usually carry several clusters of *Hcr9*s, and up to five homologues per cluster have been reported (Parniske et al., 1997; Parniske and Jones, 1999). The *Cf-9* cluster contains five homologues, *Hcr9-9A* to- *9E*, of which *Hcr9-9C* is the functional *Cf-9* gene (Parniske et al., 1997). Comparative analysis of *Hcr9* clusters suggested that point mutation, unequal recombination, gene conversion, gene duplication and translocation have contributed to the diversification of individual *Hcr9*s. Orthologous *Hcr9*s are more similar than *Hcr9* paralogues, suggesting that sequence exchange occurs most frequent between orthologues (Parniske and Jones, 1999). We previously identified the natural *Cf-9* variant *9DC* in *Lp* (chapter 2). *9DC* has the same activity and specificity in conferring HR-associated Avr9 responsiveness as *Cf-9*, and was suggested to be ancestral to *Cf-9* (chapter 2).

To date, only *Hcr9* clusters of different species, clusters with different *Cf* genes, or clusters located at different chromosomal positions have been compared to study *Hcr9* evolution (Parniske et al., 1997, 1999; Parniske and Jones, 1999). Since *9DC* and *Cf-9* are clearly related and both genes confer Avr9 responsiveness (chapter 2), we investigated the relationship between their respective clusters at the individual and population level. Isolation of the *9DC* cluster of the *Lp* accession LA1301, from which the *9DC* gene was originally isolated (chapter 2), provided us with a unique opportunity to compare the *Cf-9* and *9DC* clusters in detail. Extensive sequence homology between both clusters was revealed. We identified numerous rearrangements in the central region of the clusters that allowed us to conclude that *Cf-9* is ancestral to *9DC*. Multiple unequal recombination events have resulted in the generation of three *9DC* genes in the *9DC* cluster, which all confer Avr9 responsiveness.

Results

9DC genetics and cluster conservation

Both the *9DC* and *Cf-9* genes confer Avr9 recognition in the *Lp* population. Sequencing of seven alleles of both genes (including the previously isolated *Cf-9* allele by Jones et al., 1994) showed only three single nucleotide polymorphisms (SNPs) in *9DC* and none in *Cf-9* (chapter 2). The high DNA sequence homology (99.8%) between *9DC*, *Cf-9* and *9D*

suggested that these genes are allelic. Selfings of an F_1 of LA1301 and the susceptible cultivar MM-Cf0 showed a 3:1 segregation for HR-associated Avr9 recognition, (65:24, χ^2 = 0.18, P>0.67), which indicates that Avr9 recognition is inherited as a monogenic dominant trait in LA1301. LA1301 was crossed to cultivar MM-Cf9 (Tigchelaar, 1984), and subsequently backcrossed to MM-Cf0. All 330 BC_1 plants responded with an HR upon injection with Avr9 protein, confirming that *9DC* is allelic, or very closely linked, to *Cf-9* (< 1.8 cM, P = 95%) and located at the *Milky Way* locus (Parniske et al., 1999).

To assess the possible conservation of the *9DC* and *Cf-9* clusters in the *Lp* population, genomic DNA blots from *Lp* genotypes that either carry sequenced *9DC* or *Cf-9* alleles (chapter 2), or lack both genes, were hybridised with the *9DC* ORF probe (Figure 1). Similar hybridisation patterns may be expected if the *Hcr9* clusters are conserved in different *Lp* plants. However, only some hybridising fragments appear to be conserved, and both *Cf-9*- and *9DC*-containing *Lp* plants display variation in their *Hcr9* hybridisation patterns. Avr9-non-responsive plants show fewer *Hcr9*-hybridizing bands, as does the susceptible cultivar MM-Cf0. This suggests that the non-responsive *Lp* plants harbour fewer *Hcr9*s than Avr9-responsive plants, possibly due to a *Milky Way* cluster with only one or a few *Hcr9*s, as observed in MM-Cf0 (Parniske et al., 1997).

Isolation and assembly of the *9DC* cluster

LA1301 was chosen to molecularly characterise the *9DC* cluster, since this was also the genotype from which *9DC* was originally isolated (chapter 2), and it exhibits a strong Avr9 response. A genomic phage library with an approximate five-fold genome coverage was made from LA1301. Thirteen phages were selected based on hybridisation with a *9DC* ORF probe, and PCR-selection with *9DC*-specific primers. The inserts were subcloned in plasmids. Restriction fingerprinting, restriction hybridisation, end-sequencing of the inserts and AFLP-based fingerprinting of the clones (De Kock, Van der Hulst, De Wit and Lindhout, personal communication) of these plasmids did not result in an unambiguous contig. This suggested that *9DC* sequences are either present at two closely linked loci, or that sequence duplications are present within the *9DC* cluster. Hybridisation of a DNA blot of *Bgl*II-digested clones with a *9DC* probe enabled us to form a contig. However, this contig obscured possible duplications within the *9DC* cluster. Therefore, *Bgl*II subclones (0.7-6.4 kb) were made of selected inserts, which were sequenced by primer walking to prevent sequence assembly artefacts due to repetitive sequences. By sequencing of multiple subclones an 8.7 kb near-perfect direct repeat was detected, which interfered with contig building by conventional methods. A clone containing an insert of 16.5 kb, which almost encompassed the two complete 8.7 kb repeat regions, frequently exhibited recombination in an *E. coli* rec^- strain, which resulted in loss of part of the insert. The size of the remaining insert suggested that one repeat region was lost due to this recombination (data not shown). A similar case of

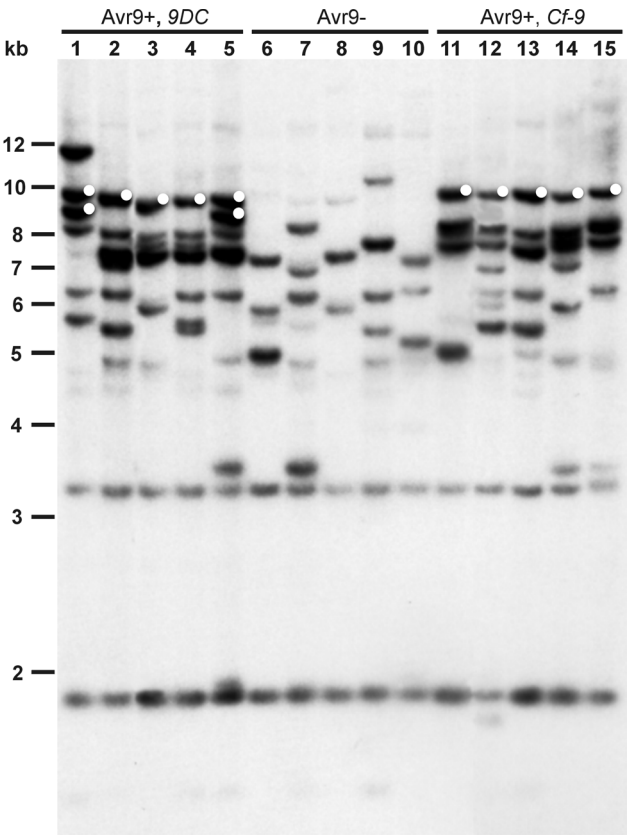

Figure 1. High stringency blot of *Eco*RV-digested genomic DNA of selected *L. pimpinellifolium* and reference genotypes, hybridised with a *9DC* ORF probe.
Lanes 1-5: LA1301, LA0114, LA1629, LA1637, LA1659 [Avr9-responsive (Avr9+), all contain sequenced 9*DC* alleles (chapter 2)]; lanes 6-10: MM-Cf0, LA0400, LA1279, LA1590, LA2852 [Avr9-non-responsive (Avr9-), contain neither *9DC* nor *Cf-9*]; lanes 11-15: MM-Cf9, LA1585, LA1614, LA1687, LA2653 [Avr9-responsive (Avr9+), all contain sequenced *Cf-9* alleles (chapter 2)]. Fragments marked with a white dot hybridise also to the *Cf-9-9D* IR region probe. A DNA size ladder is shown on the left (kb).

recombination in an *E. coli* clone leading to loss of part of a *Cf* gene cluster was previously described for the *Cf-2* cluster, which also contains repeated sequences (Dixon et al., 1996).

With the inclusion of the 8.7 kb repeat, an unambiguous contig of the *9DC* cluster was assembled that consists of 44539 base pairs (Figure 2A). The overall organisation of the cluster, including the 8.7 kb repeat, has been verified by restriction fingerprinting of individual library clones and a LA1301 DNA blot hybridisation using 14 different restriction enzymes and a *9DC* ORF probe (data not shown). The near-perfect 8.7 kb repeat includes two *9DC* genes with identical coding sequences (*9DC1* and *9DC2*, sequences described as *9DC* in chapter 2), and a third gene (*9DC3*), which is similar to *9DC1* and *9DC2*. We initially isolated clones containing either of the three *9DC* genes, as PCR with *9DC*-specific primers resulted in the same product for all three *9DC* genes. We conclude that the final contig based on these clones encompasses the complete *9DC* cluster for two reasons. First, the *9DC* cluster harbours several *LipoxygenaseC* (*LoxC*) (Heitz et al., 1997) exons, which are thought to have co-duplicated with *Hcr9*s (Parniske et al., 1997). The identity and orientation of the *LoxC* exons located at the termini of the *9DC* cluster corresponds to those found at the termini of the *Cf-4* and *Cf-9* clusters (Parniske et al., 1997). Secondly, the *9DC* cluster harbours orthologues of the most distal *Hcr9*s from the *Cf-9* cluster ('*9A*' and '*9E*', see Figure 2A).

Features of the *9DC* cluster

The *9DC* cluster contains five full-length *Hcr9*s (Figure 2A). Most striking is the presence of three *9DC* genes. The *9DC1* and *9DC2* coding sequences are completely identical and are likely the result of a duplication within the *9DC* cluster. *9DC3* is identical to *9DC1* and *9DC2* for the first 1635 nucleotides, encompassing the proposed recombination site within *9DC* (chapter 2). The remaining 3'-part has five SNPs when compared with the corresponding region in *9D* (Parniske et al., 1997), and the sequence downstream of *9DC3* is nearly identical to that downstream of *9D*, indicating that the 3'-part of *9DC3* indeed has a *9D*-like origin. '*9A*' and '*9E*' both differ only 11 bp from their respective orthologues *Hcr9-9A* and *Hcr9-9E* from the *Cf-9* cluster (Parniske et al., 1997), including a single nucleotide deletion that leads to a premature stopcodon in '*9E*'.

In addition to the complete *Hcr9*s found in the *9DC* cluster, several *Hcr9* fragments are present. The 1026 bp '*9B*' part of the '*9B9C*' fragment (Figure 2A) is 91% identical and orthologous to *Hcr9-9B* of the *Cf-9* cluster. This '*9B*' fragment is directly followed by a *Cf-9* (*9C*) fragment (position 206-821 in *Cf-9*), 831 bp of the *Cf-9* promoter directly preceding the *Cf-9* gene, and the first 821 nucleotides of the *Cf-9* ORF. Both *Cf-9* fragments carry the same SNP when compared with *Cf-9* itself. The second *Cf-9* fragment is followed by a *Cf-9* promoter fragment that starts at the same point as the first *Cf-9* promoter fragment, and merges into the *9D* promoter. This chimeric promoter precedes *9DC1*. The sequences upstream of '*9A*', downstream of ' *9E*' and the *'9A'-'9B9C*' and '*9DC3-'9E*' IRs are homologous to the corresponding regions in the *Cf-9* cluster (Figure 2A). The 6 kb IR between *9DC1* and *9DC2* and the IR between *9DC2* and *9DC3* each differ only by one single SNP from the IR between *Cf-9* and *9D* (Parniske et al., 1997), and by one SNP from each other (Figure 2A).

Conservation of *Cf-9*, *9D* and three *9DC* genes in Avr9-responsive *L. pimpinellifolium* plants

The high sequence conservation in the IRs in the *9DC* and *Cf-9* clusters and coding sequences of the three *9DC* genes and *Cf-9* indicates a clear relationship between the *Cf-9* and *9DC* clusters. Moreover, it suggests that the duplications in the *9DC* cluster are recent events. The near-identical 8.7 kb sequence in the *Cf-9* and *9DC* clusters consists of the 3' terminal half of *Cf-9*, the IR between *Cf-9* and *9D*, and the 5'-terminal half of *9D* (Figure 2B). This 8.7 kb conserved sequence could provide a means to further unravel the evolutionary relationships between the *9DC* genes and *Cf-9*. Therefore, we decided to study the occurrence of this region in the *Lp* population by PCR.

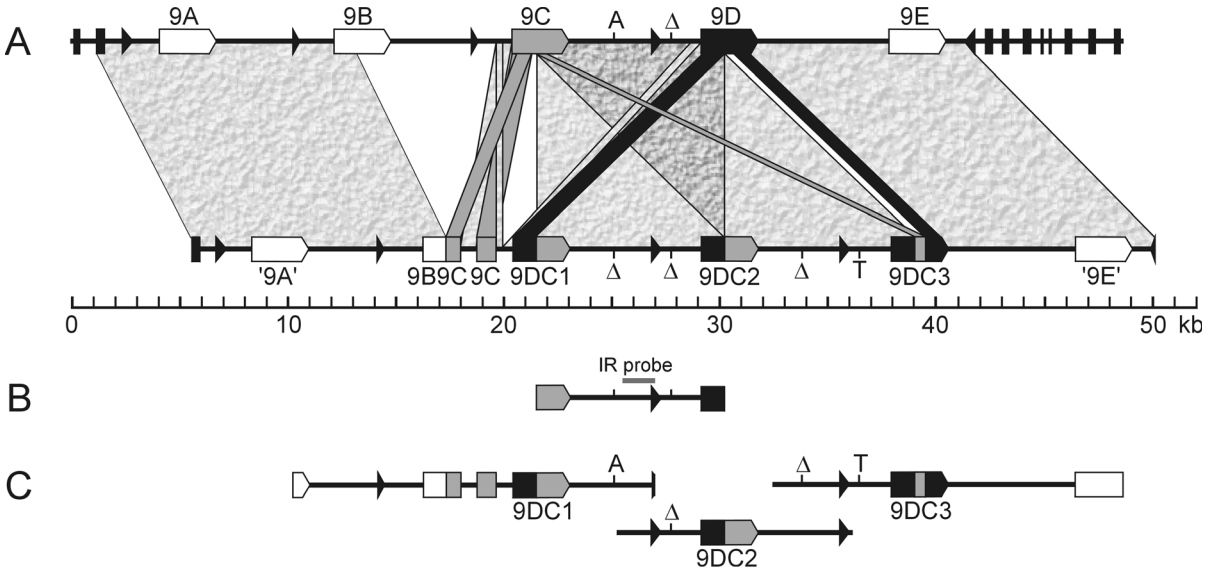

Figure 2. Schematic representation of the relationships between the *Cf-9* and *9DC* clusters, and of several genomic DNA fragments used in this study.

A. Relationships between the *Cf-9* and *9DC* clusters. The *Cf-9* cluster was previously described (Parniske et al., 1997). Arrowed boxes represent complete *Hcr9*s; rectangular boxes represent *Hcr9* pseudogenes. *Cf-9* (*9C*)-like sequences are depicted in grey; *9D*-like sequences in black; other *Hcr9* sequences in white. All *Hcr9*s and *Hcr9* fragments in the *Cf-9* and *9DC* clusters are in the 5'-3' orientation. Black arrows and bars represent *LipoxygenaseC* exons, the arrows indicate the polarity of transcription of the 3'-exon. Boxes connecting the *Cf-9* and *9DC* clusters indicate orthologous regions. Note that in the central part of the *9DC* cluster an 8.7 kb repeat is present that is near-identical to a region in the *Cf-9* cluster (see also Figure 2B). These regions are connected by light grey marbled boxes, which overlap in the dark grey marbled triangle.

B. The 8.7 kb DNA sequence fragment that is present once in the *Cf-9* cluster, and as a direct repeat in the *9DC* cluster. This fragment includes the 3'-half of *Cf-9*, the *Cf-9/9D* intergenic region and the 5'-half of *9D*. The position of the *Cf-9-9D* IR probe is indicated by a grey horizontal bar above the fragment.

C. Genomic fragments containing either of the three *9DC* genes that were cloned in a binary expression vector for agroinfiltration studies to determine their ability to confer Avr9 responsiveness.

We designed a set of specific primer pairs, based on the sequences of the three *9DC* genes, *Cf-9* and *9D*. With these primer pairs, products can only be obtained if specific combinations of the above-mentioned genes are present (Figure 3). All *9DC*- and *Cf-9*-containing genotypes appear to carry at least one tandem repeat, that comprises the 3'-half of *Cf-9*, the *Cf-9/9D* IR and the 5'-half of *9D*, whereas no PCR fragments were obtained from Avr9-non-responsive plants (Figure 3A). All *9DC* genotypes carry at least one *9DC1-9DC2* or *9DC2-9DC3* direct repeat, including the *Cf-9/9D* IR. This repeat is not present in either *Cf-9*-containing or Avr9-non-responsive genotypes (Figure 3B). Only the *Cf-9* genotypes carry a *Cf-9-9D* repeat, including the *Cf-9/9D* IR (Figure 3C).

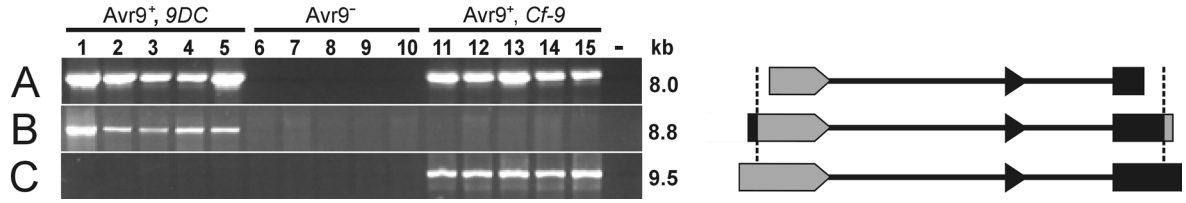

Figure 3. Presence of specific tandem repeats of the three *9DC* genes or *Cf-9* and *9D* in selected *L. pimpinellifolium* and reference genotypes, as detected by PCR.

Genotypes used for DNA templates are as shown in Figure 1. Lanes 1-5: LA1301, LA0114, LA1629, LA1637, LA1659 [Avr9-responsive (Avr9+), all contain *9DC*]; lanes 6-10: MM-Cf0, LA0400, LA1279, LA1590, LA2852 [non-Avr9-responsive (Avr9-), contain neither *9DC* nor *Cf-9*]; lanes 11-15: MM-Cf9, LA1585, LA1614, LA1687, LA2653 [Avr9-responsive (Avr9+), all contain *Cf-9*]; -: water control. On the left, PCR products are shown that were size-separated on 0.7% agarose gels. Fragment sizes were estimated using a DNA size marker (not shown). On the right, schematic representations of the structure of the PCR products are shown. *Cf-9* sequences are depicted in grey; *9D* sequences in black. The vertical hatched lines indicate the boundaries of the 8.7 kb fragment as shown in Figure 2B.

A. PCR products obtained with primers CS10 and DS13. CS10 anneals to the 3'-part of both *9DC* and *Cf-9*, DS13 to the 5'-part of all *9DC* genes and *9D*. A PCR product (8.0 kb) is obtained if a *9DC1-9DC2*, a *9DC2-9DC3* direct repeat or a *Cf-9-9D* tandem repeat is present.

B. PCR products obtained with primers DCS1 and CS1, that both anneal to the proposed recombination site (chapter 2) within all three *9DC* genes. A PCR product (8.8 kb) is obtained only when a *9DC1-9DC2* or a *9DC2-9DC3* direct repeat is present. The 17 and 15 bp fragments adjoining the 8.7 kb fragment are not drawn to scale.

C. PCR products obtained with primers CS5 and DS14. CS5 anneals to the 5'-part of *Cf-9*, and DS14 to the 3'-part of *9D*. A PCR product (9.5 kb) is obtained only when a *Cf-9-9D* tandem repeat is present.

Restriction fingerprinting of the PCR products confirmed that all fragments obtained from a specific primer pair are similar and comprise the *Cf-9/9D* IR (data not shown). In addition, control PCR experiments showed the presence of *9D* only in all *Cf-9* genotypes, and presence of *9DC3* only in all *9DC* genotypes. Conversely, in none of the *9DC* genotypes *Cf-9* is present (chapter 2; data not shown).

To confirm the PCR results and to study the structure of the *9DC* and *Cf-9* clusters in the *Lp* population, we designed a specific *Cf-9/9D* IR probe (Fig 2B). This probe was hybridised to the DNA blots used for surveying *Hcr9*-diversity in *Lp* (Figure 1). Hybridising fragments were found only in genotypes that carry *9DC* or *Cf-9* (Figure 1). The fragments that hybridised to the *Cf-9/9D* IR probe are a subset of those hybridising with the *9DC* ORF probe (Figure 1). Of the four *9DC* genotypes that we sampled in addition to LA1301, only LA1659 shows an IR hybridisation pattern identical to LA1301. The upper band in LA1301 represents the *9DC2-9DC3* IR, the lower band the *9DC1-9DC2* IR. This indicates that both a *9DC1-9DC2* and a *9DC2-9DC3* repeat are present, as in LA1301. The remaining three *9DC* genotypes only carry a *9DC2-9DC3* repeat. All *Cf-9* genotypes have a hybridisation pattern identical to that of MM-Cf9, in which only hybridisation of the *Cf-9/9D* IR is observed. Combined with the PCR data, these results indicate that all sampled *Cf-9* genotypes contain

the same *Cf-9-9D* tandem repeat. These data also show that, although the overall structure of the *Hcr9* clusters is not conserved (Figure 1), at least the genes directly downstream of *9DC* and *Cf-9* and the IRs between these genes are conserved in the *Lp* population.

Activity of individual *9DC* genes

Single amino acid changes in Cf proteins have no, or only minor effects on their activity, whereas multiple changes can lead to a drastic reduction in activity (Van der Hoorn et al., 2001a; Wulff et al., 2001). 9DC and Cf-9 have the same activity in conferring Avr9 responsiveness, although they differ in 61 amino acids (chapter 2). Compared with *9DC1* and *9DC2*, *9DC3* has 33 SNPs, resulting in only 23 amino acid substitutions. Therefore, *9DC3* may confer Avr9 responsiveness as well. This was tested by co-agroinfiltration of *9DC3* and *Avr9*. First, the activity of *9DC3* was tested in an agroinfiltration assay in tobacco with *Hcr9*s under control of the 35S promoter (Van der Hoorn et al., 2000). In this assay, in addition to *9DC1* and *9DC2*, *9DC3* is active as well (Figure 4A), as cells in the infiltrated leaf section undergo a typical HR (Van der Hoorn et al., 2000).

However, in dilution assays the activity of *9DC3* is four- to eight-fold reduced as compared to *9DC* and *Cf-9* (data not shown). As expected, *9D* from the *Cf-9* cluster (Figure 4A) and the *'9A'* and *'9E'* homologues from the *9DC* cluster did not confer Avr9 responsiveness (data not shown). An agroinfiltration assay in which *Hcr9*s are overexpressed cannot distinguish the intrinsic activities of *9DC1* and *9DC2*, which have identical coding sequences. Genomic constructs of the three *9DC* genes were therefore generated (Fig 2C), which likely reflect the intrinsic activity of *Hcr9*s in tomato plants. Surprisingly, only the genomic *9DC2* construct conferred Avr9 responsiveness in an agroinfiltration assay with Avr9 (Figure 4B). Therefore, *9DC2* is likely the main determinant of Avr9 recognition in *Lp* LA1301.

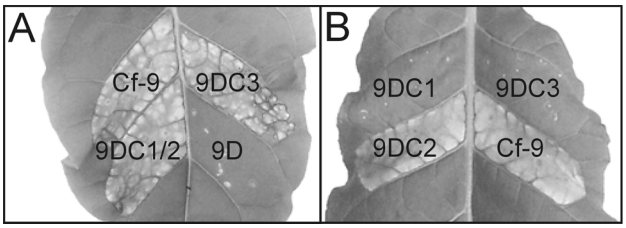

Figure 4. Combined agroinfiltration of *Avr9* under control of the 35S promoter and *Hcr9*s under control of the 35S or their native promoter.

A. Agroinfiltration of 35S-driven constructs containing the *9DC* genes, *Cf-9* or *9D*. *9DC1/2* represents the identical *9DC1* and *9DC2* coding regions.

B. Agroinfiltration assay of constructs containing *9DC1*, *9DC2* or *9DC3*, under control of their native promoter; 35S-driven *Cf-9* was used as a control. Expression of an *Hcr9* that confers Avr9-responsiveness results in visible necrosis. Both photographs were taken seven days after infiltration.

Discussion

Numerous *R* genes have been cloned in the past decade and it appears that they frequently occur in clusters (Hulbert et al., 2001; Martin et al., 2003; Takken and Joosten 2000). However, evolution of *R* gene clusters at the population level is yet poorly understood. Several studies report on the structural rearrangements in *R* gene loci (reviewed in Hulbert et al., 2001; Michelmore and Meyers, 1998). Unequal recombination, gene conversion, point mutation, duplication and translocation all contribute to the generation of novel *R* genes. The discovery of the *9DC* gene is an example of a recent event leading to a novel *R* gene (chapter 2). *Cf-9* and *9DC* are related by intragenic recombination, differ in 61 amino acids, but have a similar specificity and activity in conferring Avr9 responsiveness. Here we describe the isolation of the complete *9DC* cluster from *Lp* LA1301, and a detailed comparison with the previously isolated *Cf-9* cluster (Parniske et al., 1997). We conclude that several unequal recombination events in the *Cf-9* cluster, including two intragenic recombinations, have resulted in three *9DC* genes. Surprisingly, all three genes confer Avr9 responsiveness when overexpressed, but only *9DC2* is active under control of its native promoter. Furthermore, we discuss and reconstruct the evolution of the *9DC* cluster and show that, in contrast to what had been suggested previously (chapter 2), *Cf-9* is ancestral to *9DC*.

Reconstruction of the evolution of the *9DC* cluster

Based on previous data, it was initially assumed that *9DC* is ancestral to *Cf-9* (chapter 2). Without knowledge of the flanking regions of *9DC*, this could be explained by a single intragenic unequal recombination between *9DC* and an unidentified homologue, which gave rise to both *Cf-9* and *9D*. However, we now show that an 8.7 kb region that comprises the 3'-half of *Cf-9*, the *Cf-9/9D* IR and the 5'-half of *9D*, and is present in the *Cf-9* cluster and duplicated in the *9DC* cluster (Figure 2A, 2B). If the sequence in the *Cf-9* cluster is ancestral, a single intragenic unequal recombination event between *9D* and *Cf-9* explains the generation of the first *9DC* gene and its flanking IR sequences (Figure 5A). Conversely, two independent unequal recombinations should have occurred at the same site in the middle of two *9DC* genes, to create both *Cf-9* and *9D* from *9DC1* and *9DC2* and two other, unknown *Hcr9*s (Figure 5B). As this is very unlikely, the sequence in the *Cf-9* cluster should represent the ancestral state. Furthermore, identification of *Cf-9* alleles from a distantly related tomato species confirms that *Cf-9* indeed is an ancient gene (chapter 4).

After establishing that *9DC* is derived from *Cf-9* and *9D*, the evolution of the *9DC* cluster could be further reconstructed. The termini of the *Cf-9* and *9DC* clusters are similar, and all major rearrangements have occurred in the central region of the clusters (Figure 2A). A single intragenic unequal recombination event in the central region of the *Cf-9* cluster (Figure 6A, cluster 1) would give rise to a cluster with *Cf-9*, a single *9DC* gene and *9D* (Figure 6A, cluster 2). This cluster harbours two identical *Cf-9/9D* IRs and would therefore

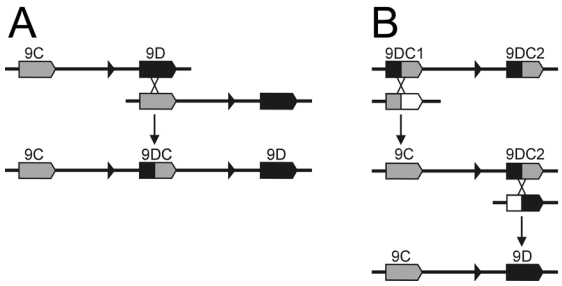

Figure 5. Schematic representation of the two possible ancestral relationships between *Cf-9* (*9C*), *9D* and *9DC*. Arrowed boxes represent complete *Hcr9*s; *Cf-9* sequences are depicted in grey; *9D* sequences in black; open boxes represent unknown *Hcr9* sequences; black triangles represent *LipoxygenaseC* exons. Diagonal crossing lines indicate (unequal) recombination points; arrows point towards one of the possible recombination products. All recombinations are shown in a homozygous genotype.
A. A single intragenic recombination between *9D* and *Cf-9* could result in the *9DC* gene.
B. The *Cf-9* and *9D* genes can only be generated from two *9DC* genes via two independent unequal recombinations at identical positions within each of these *9DC* genes. Recombination of *9DC1* with an unknown *Hcr9* that carries 5' *Cf-9*-like sequences would result in *Cf-9*. Recombination between *9DC2* and an unknown *Hcr9* that carries 3' *9D*-like sequences would generate *9D*.

be prone to further intragenic unequal recombination due to mispairing of individual homologues. Indeed, a second *9DC* copy was generated (Figure 6A, cluster 3). Two scenarios may explain the presence of a third *9DC* gene in some of the *9DC* clusters (Figure 6A, 6B). In scenario A (Figure 6A), *9DC3* was generated before *9DC1*. Intragenic recombination between the second *9DC* gene and the *9D* orthologue of cluster 3 (Figure 6A) generated a cluster that contains the *9DC2* gene and *9DC3* (Figure 6A, cluster 4A), which is present in three of the *L. pimpinellifolium* genotypes studied here. A final unequal recombination event generated a cluster that contains the two identical *9DC1* and *9DC2* genes, and *9DC3* (Figure 6A, cluster 5A), which is present in two of these *L. pimpinellifolium* genotypes, including LA1301. Alternatively, in scenario B (Figure 6B), all genotypes first accumulated the three *9DC* genes, and some have subsequently lost *9DC1* or *9DC2*. A recombination point in cluster 3 (Figure 6B) different from that shown in scenario A resulted in a cluster that contains three identical *9DC* genes (Figure 6B, cluster 4B). Intragenic recombination between the third *9DC* gene and the *9D* orthologue of cluster 4B generated a cluster that contains the two identical *9DC1* and *9DC2* genes and *9DC3* (Figure 6B, cluster 5B), which is present in two of the *L. pimpinellifolium* genotypes studied here, including LA1301. A final unequal recombination event within cluster 5B resulted in loss of *9DC1*, and generated cluster 6B, which is present in three of the *L. pimpinellifolium* genotypes studied here.

In all five tested *9DC* genotypes, *Cf-9* and *9D* have been lost. *9D* was lost through unequal recombination with *9DC2*, yielding *9DC3* (Figure 6A, 6B). The sequences upstream of *9DC1* that comprise the '*9B9C'* fragment, the two *Cf-9* fragments and two *Cf-9* promoter sequences can only be explained by numerous recombinations. These at least comprise truncation of *Cf-9* due to unequal recombination with the *Cf-9* promoter, duplication of this

Cf-9 fragment-*Cf-9* promoter sequence, fusion of the first *Cf-9* fragment with an *Hcr9-9B* orthologue, which would yield the *'9B9C'* fragment, and fusion of the second *Cf-9* promoter fragment with the *9DC1* promoter, which would yield the short chimeric *Cf-9/9D* promoter.

Mispairing of *R* gene homologues is known from several *R* gene families (Hulbert et al., 2001). Inter- and intragenic recombinations give rise to novel *R* gene homologues and novel combinations of *R* gene homologues, and thus contribute to diversity at *R* gene loci. However, if the unequal recombination rate within an *R* cluster is too high, this may lead to homogenisation of the *R* gene sequences. Consistent with this idea, it was previously suggested that sequence exchange between orthologous *Hcr9*s occurs more frequent than between paralogues (Parniske and Jones, 1999). However, the generation of three *9DC* genes from a cluster that contained only a single *9DC* gene by mispairing of, and unequal recombination between homologues is unprecedented and suggests that the initial *9DC* cluster was unstable due to the presence of the 8.7 kb repeat. In contrast, *Cf-9* was found to be very stable in a homozygous background, whereas the meiotic stability of *Cf-9* was dramatically reduced in a *Cf-4/9* heterozygous background (Parniske et al., 1997). This indicated that unequal mispairing in *Hcr9*-clusters in a homozygous background is rare, which can be explained by diverged IRs that prevent mispairing of homologues and subsequent homogenisation of the homologues. The repetitive IR structure of the initial *9DC* cluster (Figure 6A), however, appears to be prone to mispairing. This resulted in unequal recombination and the generation of three *9DC* genes. *L. pimpinellifolium* is a facultative outcrosser (Rick et al., 1977), and therefore, the *9DC* cluster could be present in heterozygous plants. Therefore, sequence exchange by inter- and intragenic recombination and gene conversion between the *9DC* genes and homologues that occupy orthologous positions may lead to further *Hcr9* sequence homogenisation, and a decrease of *Hcr9* variation at the *MW* locus.

The termini of the *9DC* cluster (5' of the '*9B*' fragment and 3' of *9DC3*) are similar to those of the *Cf-9* cluster (Figure 2A). The ORFs of the '*9A*' and '*9E*' genes each differ only by 11 bp from their paralogues *Hcr9-9A* and *Hcr9-9E* of the *Cf-9* cluster, whereas the intergenic regions show a higher proportion of SNPs, as well as some insertions and deletions. In contrast, the 8.7 kb repeat regions in the *9DC* cluster, including IRs of almost 6 kb, differ only by one or two SNPs from the corresponding region in the *Cf-9* cluster, which suggests that the formation of the three *9DC* genes in the *9DC* cluster is a relatively recent event. It further suggests that gene conversion and/or intergenic recombination have occurred at the termini of the *Cf-9* and *9DC* clusters. However, the termini of the *Cf-9* cluster may not be ancestral to those of the *9DC* cluster, but more likely represent variation in the *Hcr9-9A*-like and *Hcr9-9E*-like homologues in the *L. pimpinellifolium* population.

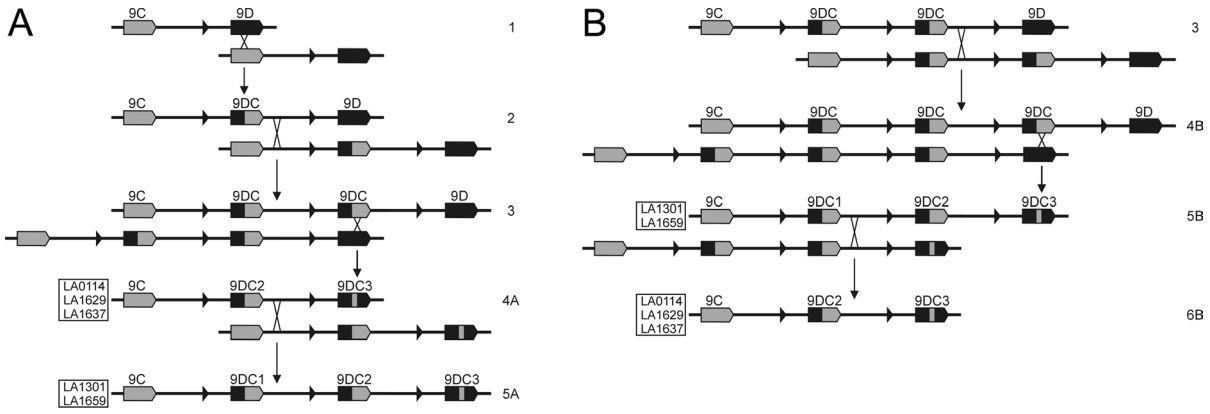

Figure 6. Schematic representation of two scenarios for the formation of the central region of the *9DC* cluster, which involve unequal recombinations due to mispairing of individual *Hcr9*s.

Arrowed boxes represent complete *Hcr9*s; *Cf-9* (*9C*) sequences are depicted in grey; *9D* sequences in black; black triangles represent *LipoxygenaseC* exons. Diagonal crossing lines indicate (unequal) recombination points; arrows point towards one of the possible recombination products. All recombinations are shown in a homozygous genotype. Only the positions of the intragenic recombination sites that generated the first of the two identical *9DC* genes and *9DC3* are known exactly. The other recombination sites may be located anywhere within the co-linear sequence stretches, and are drawn at arbitrary positions within these stretches. Boxed LA numbers to the left of clusters indicate the *L. pimpinellifolium* accessions that contain these clusters. To the right, numbers indicate the different clusters. The first three clusters are identical in both scenarios.

A. Scenario A, in which *9DC3* was generated prior to *9DC1*.

B. Scenario B, in which *9DC3* was generated after *9DC1*.

Note: recombinations that may lead to deletion of *Cf-9* or the formation of the *Cf-9* fragments present in the *9DC* cluster of LA1301 are not indicated.

Activity of *Hcr9*s mediating Avr9 recognition in *L. pimpinellifolium*

In an agroinfiltration assay, all three *9DC* genes confer Avr9 responsiveness under control of the 35S promoter, although the activity of *9DC3* is four- to eight-fold reduced when compared to *Cf-9*, *9DC1* and *9DC2*. Previously, only the two *Cf-2* genes from the *Cf-2* cluster were found to have the same function in conferring resistance to *C. fulvum* strains that express the Avr2 protein (Dixon et al., 1996; Luderer et al., 2002), and therefore, the three *9DC* genes that share the same function represent a unique situation among all known *C. fulvum* resistance gene clusters. In an agroinfiltration assay with genomic constructs, however, only *9DC2* conferred Avr9-responsiveness. Thus, *9DC2* is likely the main determinant of Avr9 recognition in *Lp* LA1301. Since *9DC1* and *9DC2* have identical downstream sequences, but *9DC1* has a promoter region of only 797 bp, the observed difference in activity may be attributed to a lower expression level of *9DC1*. Unfortunately, this cannot be verified in LA1301 plants, as both genes and their 5'- and 3'-untranslated regions do not contain any SNPs that would enable a discriminatory RT-PCR analysis. *9DC3* has the same promoter as *9DC2*, but the terminators of these two genes differ considerably. Therefore, its inactivity when expressed by agroinfiltration under its native promoter may be explained by a combination of the lower activity of the 9DC3 protein and a lower *9DC3*

expression level. The agroinfiltration assay with genomic constructs, however, is less sensitive compared to that with overexpression constructs, and therefore does not exclude that, in addition to *9DC2*, *9DC1* and/or *9DC3* may also be active in Avr9 recognition upon *C. fulvum* infection of LA1301. Unfortunately, the intolerance of LA1301 to the high humidity used in the standard infection assay prevented successful *C. fulvum* infections.

Avr9 recognition in tomato

All isolates of *C. fulvum* collected to date originate from commercially grown tomatoes. At least one of these strains can overcome the resistance provided by the *Cf-9* cluster without an apparent loss of pathogenic fitness (Laugé et al., 1998a). Moreover, *Avr9* gene replacement did not affect the pathogenic fitness of *C. fulvum* in greenhouse infection assays (Marmeisse et al., 1993). This suggests that, at least in greenhouse assays, *Avr9* may be dispensable. However, Avr9 recognition is present in a high proportion of *Lp* plants and based on the highly conserved *Cf-9* and *9DC* genes (Laugé et al., 2000; chapter 2). Avr9 recognition is also present in several other tomato species and functional *Cf-9* alleles have also been identified in a distantly related tomato species (chapter 4). This suggests that in wild tomato plants *Hcr9*s that confer Avr9 recognition may have been maintained by selection. Therefore, it would be highly interesting to collect *C. fulvum* strains from wild tomato plants and characterise both the *Avr* and *R* genes. This would add greatly to our knowledge of natural selection and co-evolution in plant-pathogen populations (Bergelson et al., 2001; DeMeaux and Mitchell-Olds, 2003; Thrall and Burdon, 2003). It may also shed light on the relative importance of the different *C. fulvum* Avr factors within the *C. fulvum* population in a natural situation, including Avr9.

Our present study enabled a unique detailed reconstruction of the evolution of a single *R* gene cluster at the population level, and has shown that unequal recombination can have a major impact on the evolution of *R* gene clusters. A great challenge in the near future will be to study *R* gene clusters at an even larger scale, by using novel *R* gene cluster fingerprinting methods.

Materials and methods

Plant material

Accessions of *Lp* were donated by the C.M. Rick Tomato Genetics Resource Center of the University of California, Davis (http://tgrc.ucdavis.edu/). The tomato cultivar MoneyMaker (MM) and the near-isogenic line MM-Cf9 (Tigchelaar 1984), which contains the *Cf-9* cluster (Parniske et al., 1997), were used as controls. Plants were grown under standard greenhouse conditions. Avr9-responsive plants were selected by injection of leaflets with Avr9 protein (10 µg/ml), and screened for visible necrosis.

DNA manipulations

DNA manipulations were performed according to standard protocols (Sambrook and Russell, 2001). DNA sequence analysis was performed using Lasergene software programs (DNASTAR, Madison, WI). PCRs were

performed with AmpliTaq (Perkin-Elmer, Wellesley, MA) or the Expand High Fidelity PCR System (Roche, Basel, Switzerland) for fragments over two kb. Hybridisations were performed with ^{32}P-labeled probes (Prime-a-gene Labeling System, Promega, Madison, WI). Genomic DNA blots were hybridised either with a *9DC* ORF probe or a *Cf-9-Hcr9-9D* (*9D*) intergenic region (IR) probe, which was obtained by PCR with primers IRF and IRR on a pBluescript II SK$^-$ (Stratagene, La Jolla, CA) library clone (see below). Primers were synthesized by Sigma-Genosys (Cambridge, UK). Primer sequences are as follows (5' to 3' direction, restriction sites underlined): 9AS1: tttttccatgggttgtgtaaaacttata; H5S1: tttttccatggattgtgtagaacttgta; CS1: gccgttcaagttgggtgtt; CS5: tttccaacttacaatcccttc; CS10: aaaccagaagaacctacaatta; CS11: ccccccctgcagtcactaatatctttttcttgtgc; DS1: gagagctcaacctttacgaa; DS9: tttttccatgggttgtgtaaaacttgtg; DS12: ccccccctgcagtaattaatatctttttcttgtgc; DS13: ggaagagaggttcacttcgta; DS14: ccaagtctaacactatcaacatttc; DCS1: gttcttatcctttaacacccaac; IRF: ctaatgtatacaaagcaacaaacc; IRR: tgaagttgtgaagggaagc.

Library construction, clone selection and sequencing

Genomic DNA was isolated (Van der Beek et al., 1992) of LA1301 plants homozygous for *9DC* and was partially digested with *Sau3A*I. Fragments were cloned into the Lambda FIX® vector and packaged using the Lambda FIX® II/XhoI Partial Fill-In Vector Kit (Stratagene) and transfected to *E. coli* KW251 (Promega). Phages carrying an *Hcr9* were identified by hybridisation of plaque lift filters with a *9DC* ORF probe. On the first phage isolations, a PCR was performed with the *9DC*-specific primers DS1 and CS1 (chapter 2) to identify phages that contain *9DC*. Pure phages were obtained after two subsequent screens. Phage DNA was isolated using a plate-lysate method (Sambrook and Russell, 2001). The tomato genomic DNA inserts were cloned into the *Not*I site of pBluescript II SK$^-$ (Stratagene). End-sequences of clones were determined using the universal M13F and M13R primers. Inserts were subcloned in pBluescript II SK$^-$ and sequenced by primer walking (triple strand coverage, BaseClear, Leiden, The Netherlands). The sequence of the *9DC* cluster of *L. pimpinellifolium* LA1301 has been deposited with GenBank under accession number AY569331.

Agroinfiltration assays

Individual *Hcr9*s were amplified by PCR with gene-specific primer pairs 9AS1/CS11 (*'9A'*), DS9/DS12 (*9DC3*) and H5S11/CS11 ('*9E*') using library clones as templates. *9D* was amplified from MM-Cf9 genomic DNA with primer pair DS9/DS12. The *Hcr9*s were cloned in pRH80, sequenced (BaseClear), and subcloned in the binary plasmid pMOG800 (Van der Hoorn et al., 2000), yielding overexpression agroinfiltration constructs. The previously described *9DC* overexpression construct (chapter 2) was used for both the *9DC1* and *9DC2* genes. Genomic agroinfiltration constructs were made by subcloning of the *Not*I inserts of pBluescript II SK- clones into pBIVM2 [a pCGN1548 (McBride and Summerfelt, 1990) derivative]. Agroinfiltration assays with *Nicotiana tabacum* cv. Petite Havana SR1 were performed as described, with use of the pAvr9 and pCf9 constructs (Van der Hoorn et al., 2000), except that *Agrobacterium tumefaciens* strain GV3101 was used. To test the relative activities of *Cf*-genes, agroinfiltration dilution series were performed as described previously (Van der Hoorn et al., 2001a).

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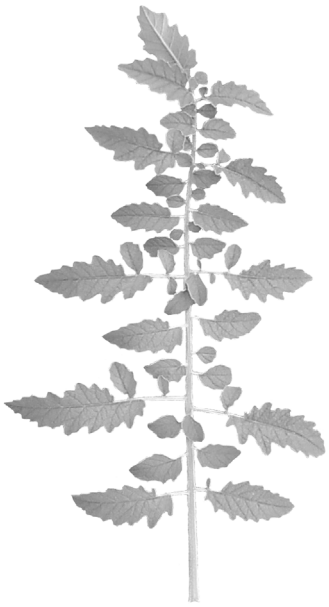
Chapter 4

The *Cf-4* and *Cf-9* resistance genes against *Cladosporium fulvum* are conserved throughout the tomato genus



Marco Kruijt, Diana J. Kip, Matthieu H.A.J. Joosten,
Bas F. Brandwagt and Pierre J.G.M. De Wit
Submitted for publication.

Resistance genes against *Cladosporium fulvum* are conserved throughout the tomato genus

Abstract

The *Cladosporium fulvum* resistance genes *Cf-4* and *Cf-9* have been introgressed into cultivated tomato (*Lycopersicon esculentum*) and confer resistance to *C. fulvum* strains that secrete the Avr4 and Avr9 elicitor proteins. Homologues of *Cf-4* and *Cf-9* (*Hcr9*s) are located in clusters and evolve mainly via sequence exchange between homologues. To study the evolution of *Cf* genes that confer recognition of the *C. fulvum* Avr4 and Avr9 elicitor proteins, we set out to identify functional homologues of *Cf-4* and *Cf-9* in wild tomato species. Plants responsive to the *C. fulvum* Avr4 and Avr9 elicitor proteins were identified throughout the *Lycopersicon* genus. Open reading frames of *Hcr9*s of Avr4- and Avr9-responsive tomato plants were PCR-amplified. Several *Cf-4* and *Cf-9* homologues of diverged tomato species, designated *Hcr9-Avr4*s and *Hcr9-Avr9*s, were identified by agroinfiltration assays. The studied *Hcr9-Avr4*s and the *Hcr9-Avr9*s are highly conserved. Domains essential for Cf-4 function are conserved in the Hcr9-Avr4s, whereas the high conservation of the Hcr9-Avr9s compared to Cf-9 is consistent with previous findings that specificity for Avr9 recognition in Cf-9 is scattered throughout the protein. We conclude that the *Hcr9-Avr4*s and *Hcr9-Avr9*s that mediate Avr4 and Avr9 recognition predate *Lycopersicon* speciation. These results further suggest that *C. fulvum* is an ancient pathogen of the *Lycopersicon* genus. Therefore, the *Hcr9-Avr4*s and *Hcr9-Avr9*s may have been maintained in the *Lycopersicon* genus by selection pressure imposed by *C. fulvum*.

Plants are continuously challenged by a wide variety of pathogens in their natural habitats. Nevertheless, most plants are resistant to these pathogens. One way plants can achieve resistance is described by the gene-for-gene model (Flor, 1946). In this model, the pathogen contains an avirulence (*Avr*) gene and the plant carries a matching resistance (*R*) gene, that mediates specific recognition of the *Avr* gene product and thereby of the pathogen itself. Upon recognition, the plant mounts various defence responses that often includes a hypersensitive response (HR), and thereby restricts pathogen growth (Hammond-Kosack and Jones, 1996). The presence of *R* genes in host plants imposes selection pressure on their pathogens to inactivate the corresponding *Avr* genes by mutation or deletion in order to gain virulence. Plants in turn, are under pressure to maintain *R* genes or generate novel *R* genes that enable them to recognise new strains of the pathogen and thereby to remain resistant.

Cladosporium fulvum is a biotrophic leaf pathogen of the tomato (*Lycopersicon*) genus. The *C. fulvum*-tomato interaction is an ideal gene-for-gene pathosystem to study plant-

pathogen interactions (Joosten and De Wit, 1999; Rivas and Thomas, 2002) and *R* gene evolution (Parniske et al., 1997, 1999; Parniske and Jones, 1999; chapters 2 and 3). Upon infection, *C. fulvum* secretes various small proteins into the apoplast of the tomato leaf that act as elicitors of defence in tomato genotypes that carry the corresponding *C. fulvum* resistance genes (*Cf* genes) (reviewed in Joosten and De Wit, 1999). Especially the Avr4 and Avr9 elicitor proteins have been extensively studied. Avr4 was shown to bind chitin oligomers and may protect the cell wall of *C. fulvum* against plant chitinases during infection of tomato leaves, or against chitinases of hyperparasitic fungi (Van den Burg et al., 2003). Avr9 is structurally related to other small cysteine-rich peptides, such as serine proteases, ion channel blockers and growth factors, and is most to related carboxy peptidase inhibitor (Vervoort et al., 1997). Although no other function besides avirulence has been reported for Avr9 yet, the homology with other proteins suggests that it may interfere with natural regulatory processes in the tomato leaf. In the *L. pimpinellifolium* population most known *C. fulvum* elicitors are recognised by certain accessions (Laugé et al., 2000), and Avr9 recognition was found in 72 out of 231 *L. pimpinellifolium* accessions tested (chapter 2). These data suggest that within this wild tomato species many different *Cf* genes exist.

Cf genes encode membrane-bound proteins that carry extracellular leucine-rich repeats (LRRs) and do not have an obvious cytoplasmic signalling domain. The *Cf-2*, *Cf-4*, *Cf-4E*, *Cf-5* and *Cf-9* genes have all been isolated from near-isogenic lines of the tomato cultivar MoneyMaker (MM) (Boukema, 1981; Dixon et al., 1996; 1998; Jones et al., 1994; Takken et al., 1999; Thomas et al., 1997; Tigchelaar, 1984). The *Cf-4* gene from *L. hirsutum* and the *Cf-9* gene from *L. pimpinellifolium* have been studied extensively, as *Cf-9* was the first isolated *Cf* gene (Jones et al., 1994), and *Cf-4* is highly homologous to *Cf-9* (Thomas et al., 1997). *Cf-4*, *Cf-4E* and *Cf-9* are all members of the *Hcr9* (Homologue of *Cladosporium fulvum* resistance gene *Cf-9*) gene family, whereas *Cf-2* and *Cf-5* are members of the *Hcr2* gene family. Clusters containing up to five *Hcr9*s are found at several loci on the short arm of chromosome 1 of tomato, and 19 different *Hcr9*s have been published to date (Jones et al., 1994; Parniske et al., 1997; Parniske and Jones, 1999; Thomas et al., 1997; chapter 2). Most of these *Hcr9*s do not confer resistance to known strains of *C. fulvum*. However, sequence exchange between homologues was shown to contribute greatly to *Hcr9* diversity (Parniske et al., 1997; Parniske and Jones, 1999; chapter 3), and may result in the generation of novel *Cf* genes. The *9DC* gene from *L. pimpinellifolium* is composed of the 5' half of *Hcr9-9D* from the *Cf-9* cluster and the 3' half of *Cf-9*, and has a similar activity and specificity in mediating Avr9 responsiveness as *Cf-9* (chapter 2). The isolation of the complete *9DC* cluster from *L. pimpinellifolium* LA1301, which contains three *9DC* genes, showed that the *9DC* gene is the result of intragenic recombination between *Cf-9* and *Hcr9-9D*. Although the precise mechanism of Avr recognition by Cf proteins is not known yet, several studies have reported specific features in Cf-4 and Cf-9 that are required for their function (Van der Hoorn et al., 2001a; Wulff et al., 2001). Furthermore, these experiments, as well as studies on the naturally

occurring *9DC* gene, showed that considerable variation in *Cf* genes is allowed without loss of function.

Although much is know of the genetic mechanisms involved in the evolution of *Hcr9*s, it is not known how *Cf* genes are maintained in wild tomato populations. The birth-and-death model (Michelmore and Meyers, 1998) describes the evolution of *R* genes that comply with the arms race model (Dawkins and Krebs, 1979). This model implies that an individual *R* gene emerges in a plant population and will be lost when the resistance is broken. The *Rps4* gene from *Arabidopsis thaliana* may have evolved according to this arms race model (Bergelson et al., 2001). Several recent studies, however, suggest that resistance genes can be maintained in plant populations over long periods of time (Mauricio et al., 2003; Riely and Martin, 2001; Rose et al., 2004; Stahl et al., 1999; Tian et al., 2002; chapter 2). Selection for *Hcr9*s that confer resistance to *C. fulvum* could maintain these genes in the *Lycopersicon* population, resulting in ancient *Cf* genes. Two *R* genes in soybean and *Arabidopsis thaliana* have evolved independently to recognise the AvrB elicitor from *Pseudomonas syringae* (Ashfield et al., 2004), showing that convergent evolution may also lead to *R* genes which mediate recognition of the same Avr protein. The versatile nature of *Hcr9* clusters and the fact that large variation in *Cf* genes is allowed without loss of function implies that convergent evolution could have resulted in independently evolved *Hcr9*s with the same specificity for a certain *C. fulvum* elicitor.

To find out which of these models applies to the *Hcr9*s, we set out to study how *Hcr9*s that mediate recognition of the *C. fulvum* elicitors Avr4 and Avr9 have evolved. We describe the identification of functional homologues of *Cf-4* and *Cf-9* of diverged tomato species. We conclude that the *Hcr9*s that confer Avr4 or Avr9 recognition predate *Lycopersicon* speciation, which also suggests that *C. fulvum* could be an ancient pathogen of *Lycopersicon* species.

Results

Avr4 and Avr9 recognition is found throughout the *Lycopersicon* genus

A genus-wide screen has been performed to identify wild tomato plants that recognise the two best-characterised *C. fulvum* elicitors, Avr4 and Avr9. Representative accessions collected throughout the natural range of each of the eight wild tomato species were screened. *L. esculentum* var. *cerasiforme* was not included, as its natural geographic range is not clear and it is not clearly separated from cultivated tomato (*L. esculentum*) and *L. pimpinellifolium* (Warnock, 1988, 1991). Plants were tested for Avr4 and Avr9 responsiveness by protein injections, rather than by Potato Virus X (PVX)-based expression of Avr proteins (Laugé et al., 2000). Protein injections allow large numbers of plants to be tested, as sufficient amounts of the Avr4 and Avr9 proteins were readily available, plants could be injected multiple times and with several proteins simultaneously, and the procedure is non-destructive. The presence

of a *Cf* gene will result in visible necrosis (HR) throughout the injected leaf section (Figure 1). Four to six plants of each accession were injected. Most accessions that contained Avr4- or Avr9-responsive plants also contained non-responsive plants, implying heterozygosity for recognition of Avr proteins within accessions. Only some Avr9-responsive *L. pimpinellifolium* accessions did not exhibit this heterozygosity. With these protein injections, additional Avr9-responsive plants in the *L. pimpinellifolium* accessions were identified, as compared to a PVX-based screen (Laugé et al., 2000; see also chapter 2).

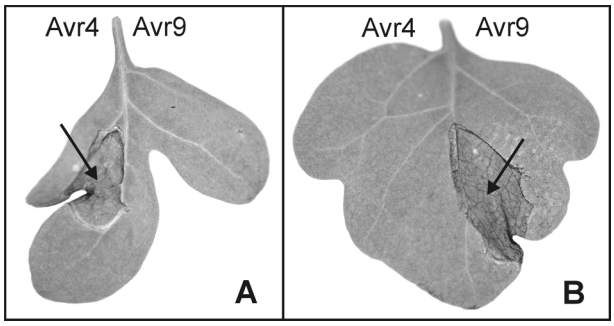


Figure 1. Examples of a specific hypersensitive response in wild *Lycopersicon* plants upon injection of the *C. fulvum* Avr4 and Avr9 elicitor proteins.
A. Primary leaf of an Avr4-responsive *L. parviflorum* LA2190 plant.
B.Primary leaf of an Avr9-responsive *L. hirsutum* LA2155 plant.
Sections of both leaves were injected with Avr4 (left side) and Avr9 (right side). Arrows indicate the visible necrotic sections. Pictures were taken four days after injection.

In Figure 2, a schematic overview of the presumed phylogenetic relationships between the eight wild tomato species in relation to the distribution of Avr4 and Avr9 recognition within 188 accessions of these species is shown. Avr4 recognition was observed throughout the *Lycopersicon* genus, with the exception of *L. pimpinellifolium* (consistent with Laugé et al., 2000). In addition to the known Avr9 recognition in *L. pimpinellifolium*, Avr9 recognition was also found in *L. hirsutum*, *L. peruvianum* (both in a northern and a southern accession), and *L. pennellii* (Figure 2).

Isolation of novel *Hcr9*s that mediate Avr4 or Avr9 recognition

Cf-4 and *Cf-9* are both members of the well-studied *Hcr9* family. Therefore, a PCR-based method for the isolation of novel *Hcr9*s that mediate Avr4 or Avr9 recognition was developed, based on sequence information of all published *Hcr9*s (Jones, et al., 1994; Parniske et al., 1997; Parniske and Jones, 1999; Thomas et al., 1997; chapter 2) and three *Hcr9*s located at the Cf-Ecp2 *Orion* locus (De Kock et al., unpublished data).

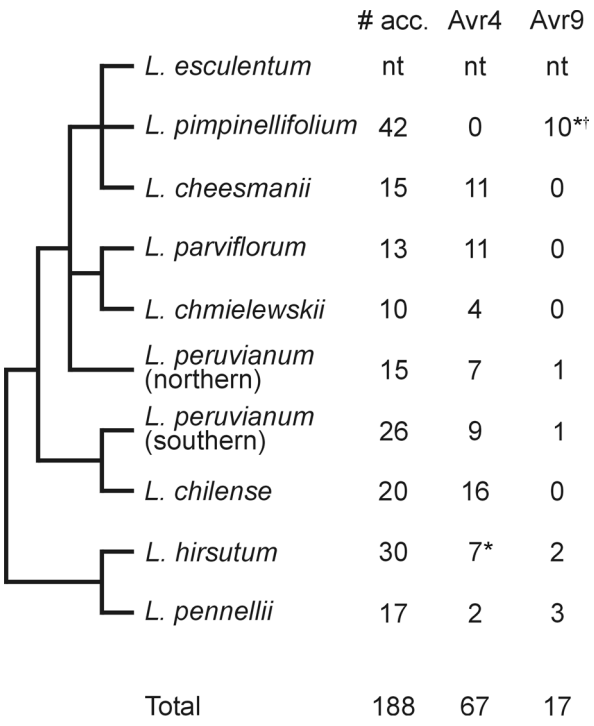


Figure 2. Tentative representation of phylogenetic relationships between the *Lycopersicon* species and Avr4 and Avr9 responsiveness within these species.
This figure does not represent a true phylogenetic tree, but is a synthesis of inter-species crossability, morphological data and phylogeny of nuclear genes (Alvarez et al., 2001; Marshall et al., 2001; Miller and Tanksley, 1990; Nesbitt and Tanksley, 2002; Peralta et al., 2001; Rick, 1979). Four to six plants of each accession were injected with the Avr4 and Avr9 elicitor proteins and scored for necrosis (hypersensitive response) two to four days after injection. The total number of accessions injected with Avr4 and Avr9 protein per species (# acc.) and the number of accessions per species responding to the Avr4 or Avr9 injections are indicated. *: Avr4 and Avr9 recognition in cultivated tomato (*L. esculentum*) has been introgressed from the wild tomato species *L. hirsutum* (*Cf-4*) and *L. pimpinellifolium* (*Cf-9*), but *L. esculentum* was not tested (nt) in this study. †: *L. pimpinellifolium* has previously been screened for Avr9 recognition (chapter 2).

We assumed that recognition of Avr4 and Avr9 in the wild tomato species is mediated by *Hcr9*s, and that these *Hcr9* could be PCR-amplified. A balanced mix of degenerate *Hcr9* primers (with *Nco*I and *Pst*I sites) was developed (Figure 3A) to maximise the number of different open reading frames (ORFs) amplified from each tomato genotype and to allow cloning of these *Hcr9*s. Patterns of nucleotides were incorporated in separate primers, to prevent high degeneracy in each primer (Figure 3A). Furthermore, the PCR conditions were optimised to yield high quantities of PCR products, using control genomic DNA preparations of MM-Cf4, MM-Cf-9 and several randomly selected wild tomato plants as a template (Figure 3B). With this mix of *Hcr9* primers, PCR was performed on genomic DNA of Avr4- and Avr9-responsive plants. PCR generally resulted in products of about 2.6 kb, which is the length of *Hcr9*s that encode proteins with 27 LRRs, like *Cf-9*. A slightly smaller fragment of about 2.4 kb was also amplified in some cases (for example, see Figures 3B and 3C), which

likely represents *Hcr9*s that encode proteins with 25 LRRs, such as *Cf-4*. These fragments were cloned into pRH80 (Figure 3D) (Van der Hoorn et al., 2000). The 35S-*Hcr9*-terminator cassettes from these plasmids were subcloned into the binary vector pMOG800 (Honée et al., 1998). *Hcr9*s amplified from *L. pennellii*, *L. peruvianum* and *L. cheesmanii* frequently contained restriction sites used for cloning (Figure 3E). Therefore, it was impossible to clone these *Hcr9*s into pMOG800. To overcome these problems, and allow cloning of amplified *Hcr9*s in a binary vector in a single step, a modified pMOG800 plasmid containing *Asc*I and *Bam*HI cloning sites was constructed, which allowed direct cloning of *Hcr9*s amplified with degenerate primers with *Asc*I and *Bam*HI restriction sites. Although control constructs carrying *Cf-4* or *Cf-9* were functional upon agroinfiltration with *Avr4* or *Avr9* expression constructs, this cloning procedure was inefficient (data not shown). Direct cloning of *Hcr9*s into the binary vector pGreen0029 (Hellens et al., 2000) containing the 35S-terminator cassette of pRH80 proved very efficient, however, constructs carrying *Cf-4* and *Cf-9* exhibited a severely reduced activity compared to the control pMOG800 *Cf-4* and *Cf-9* constructs (data not shown). Therefore, pMOG800 was chosen for further experiments, although a two-step cloning procedure is required and the restriction sites used for subcloning are present in certain *Hcr9*s.

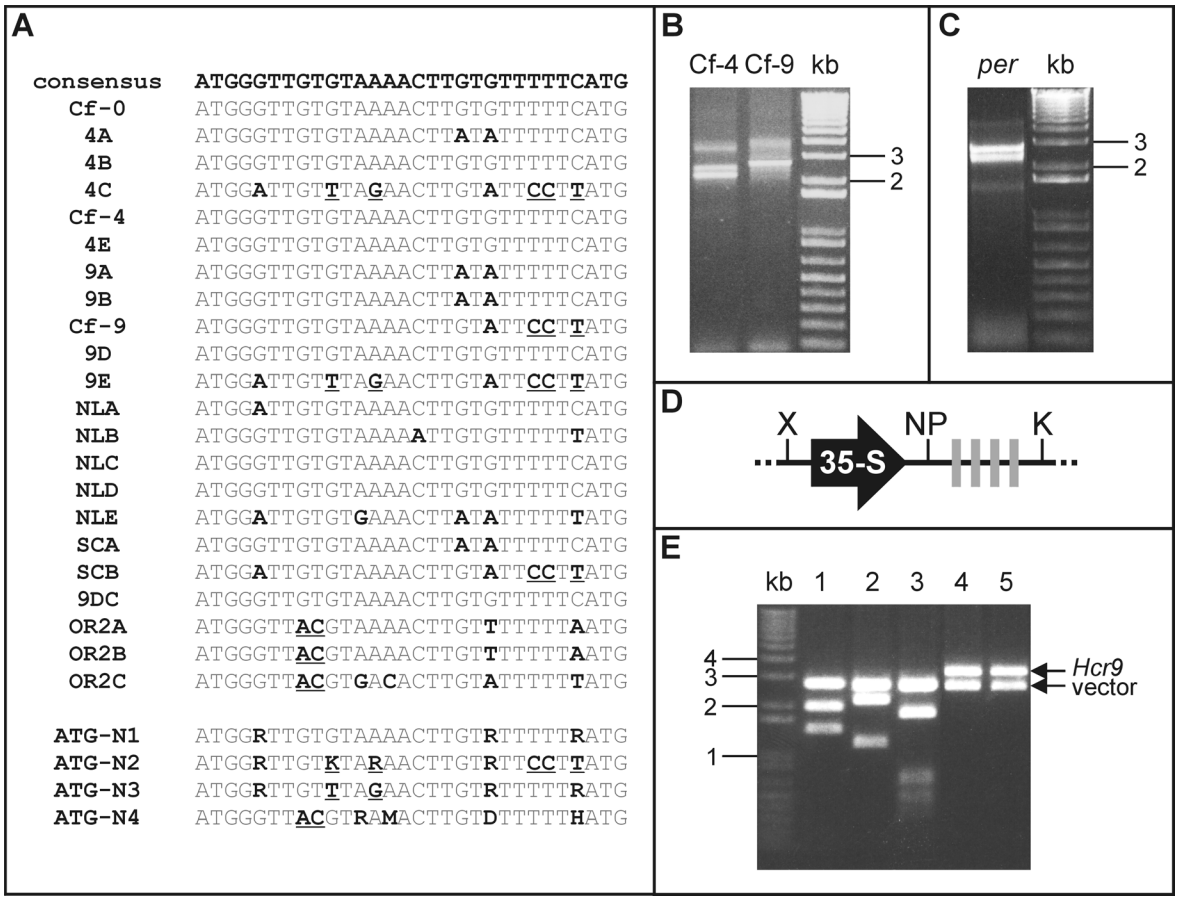


Figure 3. The method used for amplification and subsequent cloning of *Hcr9*s in the binary vector pMOG800.
A. Overview of the first 30 nucleotides of the known *Hcr9*s of the *Milky Way Cf-0*, *Cf-4*, *Cf-9*, *Northern Lights* and *Southern Cross* clusters (Parniske et al., 1997; Parniske and Jones, 1999), the *9DC* gene (chapter 2) and three *Hcr9*s from the Cf-Ecp2 *Orion* cluster (*OR2A* to *OR2C*, De Kock et al., unpublished data). Nucleotides identical to the consensus sequence are depicted in grey; polymorphic nucleotides are in boldface black. This sequence encodes the Hcr9 signal peptide, and therefore the forward primers are more degenerate than the reverse primers, which are based on the conserved 3' terminal sequence of the *Hcr9* ORF (see experimental procedures). Primers ATG-N1-N4 are the forward primers designed to amplify the maximal number of different *Hcr9*s. Nucleotide patterns present in a subset of *Hcr9*s are underlined and are present in primers ATG-N2 to -N4. Primers ATG-N1 to -N4 carry a 5' *Nco*I site, which is not depicted in this figure.
B. Example of the optimisation of the degenerate *Hcr9* PCR. PCR was performed using genomic DNA of the MM-Cf4 and MM-Cf9 cultivars, which harbour the *Cf-4* and *Cf-9* clusters, respectively. Kb indicates the lane with the DNA size marker; numbers to the right indicate fragment sizes in kilobases. Note that PCR products of about 2.4 and 2.6 kb are amplified from MM-Cf4, whereas only a 2.6 kb PCR product is amplified from MM-Cf9.
C. Example of a degenerate *Hcr9* PCR on genomic DNA of an Avr4-responsive *L. peruvianum* LA0441 plant (*per*). Note that PCR products of about 2.4 and 2.6 kb are amplified.
D. Schematic representation of the relevant part of the pRH80 plasmid (Van der Hoorn et al., 2000). The black arrow indicates the 35S promoter; four vertical grey bars indicate the TPI-II terminator. Amplified *Hcr9*s were cloned in the *Nco*I (N) and *Pst*I (P) sites of pRH80 (Van der Hoorn et al., 2000). The 35S-*Hcr9*-terminator cassette was subsequently subcloned into the binary agroinfiltration vector pMOG800 (Honée et al., 1998) using *XbaI* (X) and *Kpn*I (K).
E. Examples of *Xba*I/*Kpn*I-digested pRH80 clones. Clones 1-5 contain *Hcr9*s amplified from genomic DNA of *L. peruvianum* LA0441. For clones 4 and 5, which contain *Hcr9*s that do not contain *Xba*I and/or *Kpn*I sites, the digestion results in an intact 35S-*Hcr9*-terminator cassette fragment (indicated by *Hcr9*) of app. 3.4 kb in length, and a vector backbone fragment (indicated by vector) of app. 2.8 kb in length. The *Hcr9*s of clones 1-3 all contain *Xba*I and/or *Kpn*I sites, and therefore the 35S-*Hcr9*-terminator cassette fragment of these clones was digested into several smaller fragments and could not be subcloned into pMOG800.

Single pMOG800 clones were tested for their ability to confer Avr4 or Avr9 responsiveness using agroinfiltration in tobacco, which is a well-established tool to study *Cf* gene activity. (Thomas et al., 2000; Van der Hoorn et al., 2000, 2001a; Wulff et al., 2001; chapter 2). Following this approach, *Hcr9*s that mediate Avr4 responsiveness (designated *Hcr9-Avr4*s) were isolated, and subsequently sequenced, from *L. chilense* LA2759, *L. chmielewskii* LA1316, *L. hirsutum* LA0361, *L. parviflorum* LA2190 and (northern) *L. peruvianum* LA0441 (Table 1). Unfortunately, *Hcr9-Avr4*s could not be isolated from *L. cheesmanii* and *L. pennellii*, possibly due to the presence of restriction sites used for cloning in these *Hcr9-Avr4*s. Likewise, *Hcr9*s that mediate Avr9 recognition (designated *Hcr9-Avr9*s) could be isolated and sequenced from two different *L. hirsutum* accessions, but not from Avr9-responsive *L. pennellii* and *L. peruvianum* accessions. From a single *L. hirsutum* LA2155 plant, three different *Hcr9-Avr9*s were isolated (Table 1). *Hcr9-Avr9*s from *L. pimpinellifolium*, including *Cf-9*, *9DC* and a *9DC* variant (*9DC3*), have been described previously (Jones et al., 1994; chapters 2 and 3).

Features and activities of the *Hcr9-Avr4*s

To date, *Cf-4* was the only known *Hcr9* that mediates recognition of Avr4 and resistance to *C. fulvum* strains that produce Avr4 (Thomas et al., 1997). In this study, five additional *Hcr9-Avr4*s were isolated from five different tomato species, including *Hcr9-Avr4-hir1* from *L. hirsutum* which is identical to *Cf-4* (Table 1).

Table 1. Overview of all *Hcr9-Avr4*s and *Hcr9-Avr9s* and their relative activities.

Lycopersicon accession	*Hcr9*	Relative activity[a]
L. hirsutum LA0361	*Hcr9-Avr4-hir1* (*Cf-4*) [b]	+++
L. chilense LA2759	*Hcr9-Avr4-chl1*	+++(+)
L. chmielewskii LA1316	*Hcr9-Avr4-chm1*	++(+)
L. parviflorum LA2190	*Hcr9-Avr4-par1*	+++
L. peruvianum LA0441[c]	*Hcr9-Avr4-per1*	+
L. pimpinellifolium PI126915	*Cf-9*[d]	+++
L. pimpinellifolium LA1301	*9DC*[e]	+++
L. pimpinellifolium LA1301	*9DC3*[f]	+(+)
L. hirsutum LA2155	*Hcr9-Avr9-hir1*	+++
L. hirsutum LA2155	*Hcr9-Avr9-hir2*	+++
L. hirsutum LA2155	*Hcr9-Avr9-hir3*	+++
L. hirsutum LA1347	*Hcr9-Avr9-hir4*	+++

[a] Activity of *Hcr9-Avr4*s is compared to *Cf-4* in a transient agroinfiltration assay, that of *Hcr9-Avr9*s with *Cf-9*, see Figure 5 for examples. The relative activities are based on at least three independent agroinfiltration experiments.
[b] Sequence of *Hcr9-Avr4-hir1* is identical to *Cf-4* (Thomas et al., 1997).
[c] Northern accession of *L. peruvianum* (Peralta et al., 2001).
[d] Jones et al., 1994.
[e] Chapter 2.
[f] Chapter 3.

Amongst all published *Hcr9*s, DNA sequence identities are at least 73% (Jones et al., 1994; Parniske et al., 1997; Parniske and Jones, 1999; Thomas et al., 1997; chapter 2), whereas all *Hcr9-Avr4*s, including *Cf-4*, are over 96% identical. In Figure 4A, a DNA sequence alignment of all single nucleotide polymorphisms (SNPs) in the *Hcr9-Avr4*s is shown; *Cf-4* was chosen as the reference sequence. From a total of 109 SNPs among the *Hcr9-Avr4*s, 44 are found in only one of the *Hcr9-Avr4*s. 21 of these SNPs are only present in *Cf-4*. Furthermore, *Cf-4* and *Hcr9-Avr4-chl1* (the first identified *Hcr9-Avr4* from *L. chilense*) share 55 SNPs not present in the other three *Hcr9-Avr4*s, of which many are not present in the published *Hcr9*s. *Hcr9-Avr4-chm1*, *-par1* and *-per1* also share some unique SNPs. Many of the SNPs that are found in a single *Hcr9-Avr4* do not occur in the known *Hcr9*s, suggesting that most SNPs found within the five *Hcr9-Avr4*s represent novel point mutations. Moreover, the *Hcr9-Avr4*s carry conserved nucleotides at five positions that are not found in any other known *Hcr9*.

At the protein level, only 66 out of 808 amino acids were polymorphic within the five Hcr9-Avr4s (Figure 4B). 19 of these are located at putatively solvent-exposed positions,

mainly in LRRs 1-9, which are part of the variable domain of Hcr9 proteins. The remaining polymorphic amino acids are scattered throughout other protein domains.

A

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                                                                                                    11111
          1111112222234444444444444455555555555555666777777778888888889900122
       228013345236664233345578899999001235667777704815688899024566678917055 48
       178323901512794312663922601246683947051245791172773470133792353452203566
Cf-4   GCGGGGCCGTACCTAAGGGCCTTGT---TACTTTTATGGATCACTACAACTCTAAATAACGTGTCCCTAAGG
chl1   .G..C.AA..GA.G..C......A.......CG.................CGG.GC.GGTAAA.T...TG..
per1   .GCTCA..A.GATGTTCACTACC.CGATATGCGCCGAAAGCAGGCTTGTTCGGGGC.GGTAAA.....TGTA
par1   .GCTCA..A.GA.GTTCACTACC.CGATATGCGCCGAAAGCAGGCT.G.TCGGGGC.GGTAAA.....TGT.
chm1   AGCTCA..ACGA.GTTCACTACC.CGATATGCGCCGAAAGC.GGCT.G.TCGGGGCGGGTAAA..ATATGT.

       11111111111111111111111222222222222
       33344556666778888888999990011111133 33
       16944451278792233389224561902999916 99
       43148731644953534504690280004456971 19
Cf-4   GACCCATAGGGTTTCGGAAACGTATTTCG---AGGTA
chl1   ..T.........................GGTT....
per1   A..AG.CC..A.C.ATACGG.ACCGC.T....TT.AG
par1   AT..GGCC......ATACCG.AC.GC.T....TT.AG
chm1   A...G.CCAA.A.AATACGGTAC.GCATA...TTTAG
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B

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                                                 111111111112  222222222
       AABBBBB11233344444445555556788888899999013456677990LL222233345DEFG

Cf-4   FEAEVPDRIYRVSYRLN-TFKVDESIYSSSLYNDFKSRSNLPAGNTLTYFENSVLDHIVHFV-EVK
chl1   L..Q.N.SS.T...H..............VDGA.EGYT..R...I..............GD..
per1   LQSQI.NSSFTLFH.DQYSLRENRHVFGCLVDGA.EGYT..RSE..IR.SK.P.FTRVAPL..DLR
par1   LQSQI.NSSFTLFH.DQYSLRENRHVFG.LVDGA.EGYT..RS.Y..RCS....FTPVA.L..DLR
chm1   LQSQI.NSSFTLFH.DQYSLRENRPVFG.LVDGAVEGYTKMRS....R.S.K.DFTRVA.LD.DLR
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Figure 4. Alignment of *Hcr9-Avr4* DNA and protein sequences.
All sequences are compared to the reference *Cf-4* DNA (A) or protein (B) sequence of *L. hirsutum* (Thomas et al., 1997). Only residues that deviate from these sequences are shown in the alignment, residues identical to those in *Cf-4* are indicated by a dot. chl, *L. chilense*; per, *L. peruvianum*; par, (northern) *L. parviflorum*; chm, *L. chmielewskii*.
A. Nucleotide alignment of the *Hcr9-Avr4*s. The position of each polymorphic nucleotide is relative to the first nucleotide of the start codon of the *Hcr9-Avr4* consensus sequence, and is indicated by vertical numbers above each nucleotide. Dashes indicate nucleotide deletions relative to the other *Hcr9-Avr4*s. The first 30 nucleotides anneal to the forward primers, and therefore, the single nucleotide polymorphisms at positions 21 and 27 may represent PCR-induced mutations.
B. Amino acid alignment of the Hcr9-Avr4s. The positions of the polymorphic sites in the different structural domains (A-G) (Jones and Jones, 1997) are indicated above each amino acid residue. Numbers indicate individual LRRs (within domain C); L refers to the loop-out within the LRR-region. Putative solvent-exposed residues are in boldface black.

The isolated *Hcr9-Avr4*s are polymorphic compared to the previously isolated *Cf-4* and *Cf-9* genes. Therefore, their activity relative to *Cf-4* was determined by agroinfiltration. Dilution series of *Agrobacterium tumefaciens* cultures carrying the *Hcr9-Avr4* constructs were combined with a culture that carries an *Avr4* construct and infiltrated in tobacco leaves.

Their ability to confer Avr4 responsiveness was compared to that of the *Cf-4* control (Van der Hoorn et al., 2001a). The results of these experiments are summarised in Table 1, and examples of agroinfiltration dilution series with *Hcr9-Avr4-chm1* and *Hcr9-Avr4-chl1* are shown in Figure 5. *Hcr9-Avr4-per1* was less active in conferring Avr4 responsiveness than *Cf-4*. Interestingly, *Hcr9-Avr4-chm1* is slightly less active than *Cf-4* (Figure 5A), whereas *Hcr9-Avr4-chl1* is slightly more active than *Cf-4* (Figure 5B). *Hcr9-Avr4-par1* is similarly active to *Cf-4*.

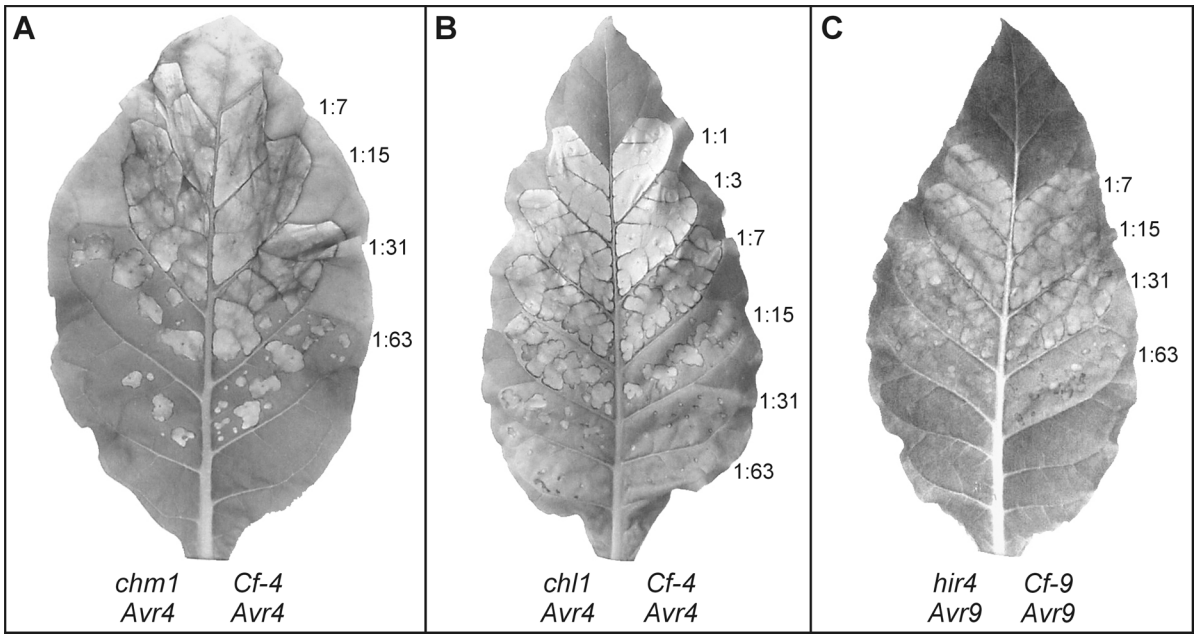

Figure 5. Examples of agroinfiltration dilution series in *N. tabacum* leaves of *Hcr9-Avr4*s and *Hcr9-Avr9s* in combination with the matching *Avr4* and *Avr9* elicitor genes, all under control of the 35S promoter.
The activity of the *Hcr9-Avr4*s was compared to *Cf-4*, and the activity of the *Hcr9-Avr9*s was compared to *Cf-9*. Dilution series were as follows: *Cf* culture:*Avr* culture ratios; 1:1; 1:3; 1:7; 1:15; 1:31; 1:63 (dilutions 1-6). Dilutions were infiltrated in separate leaf sections from top to bottom.
A. *Hcr9-Avr4-chm1* activity in conferring Avr4 responsiveness is about half of that of *Cf-4*. Dilutions 3-6 were infiltrated. Left side, *Hcr9-Avr4-chm1* with *Avr4*; right side, *Cf-4* with *Avr4*.
B. *Hcr9-Avr4-chl1* is about twice as active as *Cf-4* in conferring Avr4 responsiveness. Dilutions 1-6 were infiltrated. Left side, *Hcr9-Avr4-chl1* with *Avr4*; right side, *Cf-4* with *Avr4*.
C. *Hcr9-Avr9-hir4* is as active as *Cf-9* in conferring Avr9 responsiveness. Dilutions 3-6 were infiltrated. Left side, *Hcr9-Avr9-hir4* with *Avr9*; right side, *Cf-9* with *Avr9*.

Features and activities of the *Hcr9-Avr9*s

Seven identical *L. pimpinellifolium Cf-9* alleles as well as seven near-identical *9DC* alleles have been isolated previously (Jones et al., 1994; chapter 2). In this study, four additional *Hcr9-Avr9*s from the distantly related tomato species *L. hirsutum* have been isolated. In Figure 6A a DNA sequence alignment of these novel *Hcr9-Avr9*s is shown; *Cf-9* was chosen as the reference sequence. Overall, the DNA sequences of all *Hcr9-Avr9*s isolated from *L.*

hirsutum are over 98% identical to *Cf-9*. Most striking is *Hcr9-Avr9-hir1* of *L. hirsutum* LA2155, which differs only by one silent mutation from *Cf-9* (SNP 177, Figure 6A), which strongly suggests a common ancestry between these two genes. From the same LA2155 plant, two additional *Hcr9-Avr9*s (*hir2* and *hir3*) were isolated, which also carry SNP 177 from *Hcr9-Avr9-hir1*. *Hcr9-Avr9-hir2* and *-hir3* are likely derived from the same ancestral *Hcr9*. Besides SNP 177, *Hcr9-Avr9-hir2* and *-hir3* differ from *Cf-9* by 13 and 15 additional SNPs, respectively, which also occur in other *Hcr9*s. SNP 177 is not found in any of the previously described *Hcr9*s, or the *Hcr9*s described in this study, which suggests it is the result of a recent point mutation that has occurred in the *Hcr9-Avr9-hir* genes. *Hcr9-Avr9-hir4* was isolated from *L. hirsutum* LA1347, and differs from *Cf-9* by 38 SNPs, including SNP 1721, which is shared by *Hcr9-Avr9-hir2* and *-hir3*. *Hcr9-Avr9-hir4* further shares the two SNPs with *Hcr9-Avr9-hir3* that distinguishes it from *Hcr9-Avr9-hir2*. Only seven of these 38 SNPs are unique to *Hcr9-Avr9-hir4*.

A

```
          1111111111111111111111111111222222222222222222
      13890233455555566777788888899999900000111113345555
      76316467837889938226905778800003600079046775621236
      76227591839045661157917684536786312317880588485524

Cf-9  CTGTCCTACAATAGCAATACTGAACGGGTTCATGGACGTATTTTACGCGA
hir1  T.................................................
hir2  T.......TCTCCATTCC.ACC............................
hir3  T.......TCTCCATTCC.ACC..................A.C.......
hir4  .AACGACC.........CT...TGAAAAACATATACTACTAACCTTATCG
```

B

```
        111111222222222    2222EFFGG
      28248999001122233LLLL4455EFFGG

Cf-9  DASSKILSMMFQMGRTEGFISLDLVEASMK
hir1  ............................
hir2  ....NSFNLLS.TR.............
hir3  ....NSFNLLS.TR.............
hir4  ETCR......SH..SAKRTFTFTFADTLIR
```

Figure 6. Alignment of *Hcr9-Avr9* DNA and protein sequences.
All sequences are compared to the reference *Cf-9* DNA (A) or protein (B) sequence of *L. pimpinellifolium* (Jones et al., 1994). Only residues that deviate from these sequences are shown in the alignment, residues identical to those in *Cf-9* are indicated by a dot. The *9DC* genes from *L. pimpinellifolium* were not included in this alignment, as single nucleotide polymorphisms in these genes relative to *Cf-9* are derived from *Hcr9-9D* by intragenic recombination between *Cf-9* and *Hcr9-9D* (chpaters 2 and 3).
A. Nucleotide alignment of the *Hcr9-Avr9*s. The position of each polymorphic nucleotide is relative to the first nucleotide of the start codon of the *Hcr9-Avr9* consensus sequence, and is indicated by vertical numbers above each nucleotide.
B. Amino acid alignment of the Hcr9-Avr9s. The positions of the polymorphic sites in the different structural domains (A-G) (Jones and Jones, 1997) are indicated above each amino acid residue. Numbers indicate individual LRRs (within domain C); L refers to the loop-out within the LRR-region. Putative solvent-exposed residues are in boldface black.

At the protein level (Figure 6B), Hcr9-Avr9-hir1 is identical to Cf-9. The identical Hcr9-Avr9-hir2 and -hir3 proteins differ only by nine amino acids from Cf-9, which are located in LRRs 18-22, of which only two are located at solvent-exposed positions (Figure 6B). The polymorphic residues of Hcr9-Avr9-hir4 relative to Cf-9 are more scattered throughout the protein (Figure 6B). Overall, the Hcr9-Avr9s carry six conserved amino acids that are not present in any other known Hcr9 protein, of which four are located at solvent-exposed positions in LRRs 7-13.

The activities of the *Hcr9-Avr9*s were determined relative to *Cf-9* by agroinfiltration of dilution series with *Avr9* (Van der Hoorn et al., 2001a). All four *Hcr9-Avr9*s from *L. hirsutum* are as active as *Cf-9* in conferring Avr9 responsiveness (Table 1), including *Hcr9-Avr9-hir4* (Figure 5C), which is most diverged from *Cf-9* (Figure 6). To date, only the *9DC3* gene from *L. pimpinellifolium* LA1301 is less active than *Cf-9* (chapter 3).

Discussion

Isolation of *Hcr9-Avr4*s and *Hcr9-Avr9*s from wild tomato species

With protein injections, Avr4 recognition was found in all *Lycopersicon* species, except for *L. pimpinellifolium*. With a PVX-based screen also no Avr4-responsive *L. pimpinellifolium* plants were identified (Laugé et al., 2000). Therefore, it is likely that Avr4 recognition is completely absent in this species. Avr9 recognition was found in only a few, but evolutionary diverged species (Figure 2). The method for isolation of *Hcr9*s that mediate recognition of the *C. fulvum* elicitors Avr4 and Avr9, from different tomato species, was shown to be successful. It allowed relative fast isolation of these *Hcr9-Avr4*s or *Hcr9-Avr9*s and therefore enabled population studies of these *Hcr9*s, which would not be feasible by map-based cloning of these genes or by screening of genomic libraries. However, this method depends heavily on known *Hcr9* sequences, which might prohibit the isolation of certain *Hcr9-Avr4*s or *Hcr9-Avr9*s.

Using the PCR-based method to isolate *Hcr9-Avr4*s and *Hcr9-Avr9*s from wild tomato plants, *Hcr9-Avr4*s from five different species were isolated, including a *Cf-4* allele from *L. hirsutum* LA0361, which is identical to the previously isolated *Cf-4* gene (Jones et al., 1994). In addition to the known *L. pimpinellifolium Hcr9-Avr9*s (*Cf-9, 9DC* and *9DC3*) (Jones et al., 1994; chapters 2 and 3), four novel *Hcr9-Avr9*s from *L. hirsutum* were isolated in this study. However, *Hcr9-Avr4*s or *Hcr9-Avr9*s could not be isolated from all Avr4- or Avr9-responsive species. Recognition of Avr4 or Avr9 in these plants could be based on another type of *R* gene, or it may be based on an *Hcr9* that could not be amplified with the degenerate primer mix. Furthermore, amplified *Hcr9-Avr4*s or *Hcr9-Avr9*s may contain restriction sites used for cloning, which precludes testing of the functionality of the *Hcr9* in the binary expression vector used in this study. For instance, amplified *Hcr9*s from *L. pennellii* frequently contained such restriction sites. *L. pennellii* occupies a diverged position

within the *Lycopersicon* genus (Figure 2) and is closely related to the genus *Solanum* (Taylor, 1986). *Hcr9-Avr4*s and *Hcr9-Avr9*s from *L. pennellii* may therefore differ from those of other *Lycopersicon* species. Altogether, the isolated *Hcr9-Avr4*s and *Hcr9-Avr9*s likely represent a part of the total variation present in genes that mediate Avr4 and Avr9 responsiveness in the *Lycopersicon* genus.

Avr4 and Avr9 recognition predate *Lycopersicon* speciation

Avr4 and Avr9 recognition is found throughout the *Lycopersicon* genus, suggesting that these are ancient traits. Moreover, the isolated *Hcr9-Avr4*s and *Hcr9-Avr9*s are highly conserved, which indicates that both the *Hcr9-Avr4*s and the *Hcr9-Avr9*s share a common origin. This further suggests that all *Hcr9-Avr4*s and *Hcr9-Avr9*s are located at the *Milky Way* (*MW*) *Cf-4/9* locus (Parniske et al., 1997, 1999). Most likely, an *Hcr9-Avr4* was present in a *Lycopersicon* ancestor at the *MW* locus before *Lycopersicon* speciation occurred. *L. hirsutum* and *L. pimpinellifolium* have been used extensively as sources of *Cf* genes, which explains why *Cf-4* and *Cf-9* are encountered in tomato breeding lines (Haanstra et al., 2000c; chapter 2), rather than other *Hcr9-Avr4*s and *Hcr9-Avr9*s. Identical *Cf-4* and *Cf-9* alleles have been isolated from different accessions of *L. hirsutum* and *L. pimpinellifolium*, respectively, as well as highly conserved *9DC* alleles (this study; chapter 2), suggesting low allelic diversity of *Hcr9-Avr4*s and *Hcr9-Avr9*s within *Lycopersicon* species. It was recently shown that the *9DC* gene has evolved from the *Cf-9* gene (chapter 3). Moreover, *Cf-9* appears to be conserved between *L. pimpinellifolium* and *L. hirsutum*. Whereas in seven *Cf-9* alleles no variation was found (Jones et al., 1994; chapter 2), the four *Cf-9* alleles from *L. hirsutum* are all different. *L. pimpinellifolium* is mostly self-compatible, whereas *L. hirsutum* is mostly self-incompatible (Rick, 1977, 1979), which may explain the higher polymorphism in the *L. hirsutum Cf-9* alleles. Therefore, it is likely that *Cf-9* was present in a *Lycopersicon* ancestor and was maintained in distinct species.

Divergence of the individual *Hcr9-Avr4*s is likely a combination of point mutations and sequence exchange by gene-conversion or recombination with other *Hcr9*s, as many of the discriminatory SNPs between the *Hcr9-Avr4*s are shared with other *Hcr9*s. Likewise, the overall high sequence conservation of the *Hcr9-Avr9*s from *L. hirsutum* and *Cf-9* from *L. pimpinellifolium* suggests that *Cf-9* was present in an ancestral *Lycopersicon* species, and has not diverged to a great extent since. The sequence polymorphisms in the *Hcr9-Avr4*s coincide with the evolutionary relationships among the *Lycopersicon* species from which these genes were isolated (Figures 2 and 4). For example, *Hcr9-Avr4-chl1* is most homologous to *Cf-4*, and *L. chilense* and *L. hirsutum* are closely related, and *Hcr9-Avr4-chm1*, *-par1* and *-per1* differ only little, which fits with the close relationship between these three species.

In the *9DC MW* cluster of *L. pimpinellifolium* LA1301 three Avr9-responsive *9DC* genes were identified (chapter 3). Similarly, two highly homologous *Cf-2* genes from the *Cf-2* cluster both confer resistance to strains of *C. fulvum* that express Avr2 (Dixon et al., 1996; Luderer et al., 2002). Three distinct *Hcr9-Avr9*s (*Hcr9-Avr9-hir1*, *-hir2* and *-hir3*) were

isolated from a single self-incompatible, and therefore heterozygous, *L. hirsutum* LA2155 plant. *Hcr9-Avr9-hir2* and *-hir3* may be the result of a duplication event similar to those found in the *9DC* and *Cf-2* clusters, and *Hcr9-Avr9-hir1* may be located at the orthologous *MW* locus. The few SNPs in the *Hcr9-Avr9*s suggest that limited sequence exchange has occurred both between these genes, and with other *Hcr9*s.

Solanum and *Lycopersicon* have a common ancestry and are highly related (Taylor, 1986), but are generally considered as separate genera. However, a minority of authors place tomatoes in the *Solanum* genus (Peralta and Spooner, 2000), which emphasises the close relationship. Avr9 recognition has also been observed in four tuber-forming *Solanum* species, upon PVX::*Avr9* infection (Laugé, personal communication). This was confirmed in *S. hannemanii* and *S. leptophyes* by Avr9 protein injections (data not shown), indicating that Avr9 recognition may be conserved between *Lycopersicon* and *Solanum*. Three linked loci with *Cf* gene homologues have been reported on the short arm of chromosome 1 of cultivated potato (*Solanum tuberosum*) (Leister et al., 1996). Moreover, in several Solanaceous species, including *S. tuberosum*, fragments that hybridise to *Cf-9* have been identified. Furthermore, *S. tuberosum* contains a high-affinity binding site for Avr9 (Kooman-Gersmann et al., 1996), and *Cf-9* is functional in conferring Avr9 responsiveness in several Solanaceous species, including *S. tuberosum* (Hammond-Kosack et al., 1998; Van der Hoorn et al., 2000). Therefore, Avr9 responsiveness in some *Solanum* species may well be based on conserved *Hcr9-Avr9*s. Several different *Hcr9*s from Avr9-responsive *Solanum* plants were amplified, these all contained restriction sites for cloning, and could therefore not be tested for Avr9 responsiveness (data not shown).

We did not find evidence for convergent evolution of the *Hcr9-Avr4*s and the *Hcr9-Avr9*s, however, this cannot be excluded, since our screen was limited to a subset of *Hcr9*s that could be amplified with the degenerate *Hcr9* primers. The isolation of additional *Hcr9-Avr4*s and *Hcr9-Avr9*s, and possibly the corresponding complete *Hcr9* clusters, would yield insight in the population structure of the *Hcr9-Avr4*s and *Hcr9-Avr9*s in individual species, as was shown for *Cf-9* and *9DC* in *L. pimpinellifolium* (chapters 2 and 3), ultimately at the *Lycopersicon* genus level, and possibly even in *Solanum*. Analysis of non-functional orthologues of *Hcr9-Avr4*s and *Hcr9-Avr9*s may also contribute to the understanding of specificity and evolution of Avr4 and Avr9 responsiveness mediated by *Hcr9*s.

Activity of the *Hcr9-Avr4*s and the *Hcr9-Avr9*s

By domain swapping and gene-shuffling experiments between *Cf-4* and *Cf-9* several features crucial for conferring recognition of Avr4 were identified in Cf-4 (Van der Hoorn et al., 2001a; Wulff et al., 2001). All four novel Hcr9-Avr4s share these essential features, including (i) 25 LRRs, a feature unique to Cf-4, (ii) a 10 amino acid deletion in the B-domain and (iii) three amino acids at solvent-exposed positions in the LRR domain of Cf-4 (Van der Hoorn et al., 2001a; Wulff *et al*, 2001). Solvent-exposed residues within the LRRs of *Hcr9*

proteins are thought to interact with other proteins (Kobe and Deisenhofer, 1994; Jones and Jones, 1997). However, no additional specific solvent-exposed residues could be identified in the *Hcr9-Avr4*s that influence their activities. In an agroinfiltration overexpression assay, the activity of *Hcr9-Avr4-per1* was reduced compared to *Cf-4*. Hcr9-Avr4-per1 contains two unique proline residues that are located in the loop-out region and at a non-solvent-exposed position in LRR 23 (Figure 4B), which may well disrupt the tertiary structure of the protein and thereby reduce its activity. Only *Hcr9-Avr4-par1* was as active as *Cf-4* in conferring Avr4 responsiveness, whereas the activities of *Hcr9-Avr4-chm1* and *Hcr9-Avr9-chl1* differ slightly from *Cf-4*. Therefore, these three *Hcr9-Avr4*s may well function in resistance against *C. fulvum* strains that express Avr4.

All *Hcr9-Avr9*s from *L. hirsutum* are as active as *Cf-9* agroinfiltration overexpression assays with Avr9. Therefore, none of the amino acid substitutions alters the activity of the Hcr9-Avr9s relative to Cf-9. The number of amino acid substitutions in the Hcr9-Avr9-hir proteins compared to Cf-9 is relatively low when compared to the amino acid substitutions found in the Hcr9-Avr4s. This suggests that sequence exchange between *Cf-9* and other *Hcr9*s may disrupt Cf-9 function, consistent with the observation that Cf-9 specificity is scattered throughout the protein (Van der Hoorn et al., 2001a; Wulff et al., 2001). However, the 9DC protein is as active as Cf-9 although it differs by 61 amino acids from Cf-9 (chapter 2). The differences in 9DC are located in the N-terminal half of the protein up to LRR 11 and are apparently not located at positions essential for conferring Avr9 responsiveness, whereas those in the Hcr9-Avr9s are mostly located in the C-terminal domains.

The *Hcr9-Avr4*s and *Hcr9-Avr9*s may be maintained by selection imposed by *C. fulvum*

A high proportion of all sampled *Lycopersicon* species and accessions specifically recognises the Avr4 or Avr9 elicitor proteins of *C. fulvum* (Figure 2), and Avr9 recognition is even found outside the *Lycopersicon* genus. Moreover, the *Hcr9-Avr4*s and *Hcr9-Avr9*s described in this study are highly conserved. Conservation of an ancient *R* gene specificity in tomato has also been demonstrated for the *Pto* gene (Riely and Martin, 2001). This suggests that tomato *R* genes are not rapidly replaced by novel ones, likely reflecting a selection pressure on the plant to maintain functional *R* genes. Not only does this suggest that Avr4 and Avr9 responsiveness is beneficial to wild tomato plants, it also suggests that *C. fulvum* is an important pathogen of these species and that Avr4 and Avr9 are beneficial to the pathogen. Maintenance of *Avr* genes in a pathogen population indicates that they contribute to virulence or saprophytic fitness (Brown, 2003; Van der Hoorn et al., 2002).

L. hirsutum, *L. pimpinellifolium*, *L. pennellii*, and *L. peruvianum* plants have been reported to be susceptible for *C. fulvum* in greenhouse infection assays (Boukema, 1981; Kerr et al., 1971; Leski, 1969). It remains to be determined whether *C. fulvum* is pathogenic on all wild *Lycopersicon* species, but in all species plants were identified that recognise either the *C. fulvum* Avr4 or Avr9 elicitor. Therefore, all *Lycopersicon* species may be host to *C. fulvum*, or to highly related pathogens that share a common ancestry with *C. fulvum* and

secrete Avr4- and Avr9-like proteins. We identified Avr9-responsive *Solanum* species, and these may be host to such *C. fulvum*-related pathogens. Alternatively, the *Hcr9-Avr4*s and *Hcr9-Avr9*s might mediate resistance to a variety of different pathogens, which produce Avr4- and Avr9-like proteins. Possibly, the Hcr9-Avr4s and Hcr9-Avr9s guard a virulence target (Van der Biezen and Jones, 1998) that is attacked by virulence proteins from different pathogens.

In conclusion, this study has broadened our understanding of the evolution of *R* genes in natural plant populations. A high proportion of wild tomato plants specifically recognises the *C. fulvum* Avr4 and Avr9 elicitors, and functional homologues of the *C. fulvum* resistance genes *Cf-4* and *Cf-9* are conserved in diverged tomato species. This suggests that these genes were present before tomato speciation occurred and that they are maintained in the tomato population, possibly due to selection imposed by *C. fulvum*. A population survey of *C. fulvum* and related pathogens on different wild *Lycopersicon* and *Solanum* species would provide further insight into the variation of *Avr* genes in natural populations of *C. fulvum*. Not only would this allow us to compare these populations to strains isolated from cultivated tomato with known *Cf* genes, but the variation in *Avr* genes in *C. fulvum* could also be compared to the variation in *Cf* genes in wild tomato species, in order to determine if the *Cf* genes in wild tomato indeed impose a selection pressure on *C. fulvum.*

Materials and methods

Plant screening and selection

Seeds of wild *Lycopersicon* accessions, consisting of the core collections of eight wild species, were kindly provided by the C. M. Rick Tomato Genetics Resource Center of the University of California, Davis (http://tgrc.ucdavis.edu/). Plants were grown in trays and pots under standard Dutch greenhouse conditions. Of each accession, four to six plants were injected with Avr4 and Avr9 protein in the first expanded leaf. Both Avr4 and Avr9 were injected with a micro-syringe (Ito Corporation, Fuji, Japan) at app. 10 µM, which is sufficient to give an HR in the control plants MM-Cf4 and MM-Cf9. Wild type Avr4 and FLAG-Avr4-S107A (a glycosylation-minus Avr4 mutant, with an N-terminal FLAG tag) were produced in *Pichia pastoris* as described in Van der Burg et al. (2001). *P. pastoris* culture filtrates containing Avr4 or FLAG-Avr4-B_{20} were dialysed against water, and subsequently purified on a G-25 column (26 mm diameter, 40 cm length, Amersham-Pharmacia, Buckinghamshire, UK). The column was eluted with water at a rate of 1 ml/minute and 5 ml fractions were collected. Fractions were tested for necrosis-inducing activity in MM-Cf4 plants and active fractions were pooled. Wild type Avr4 and FLAG-Avr4-S107A were similarly active in the leaf necrosis assay. Avr9 was obtained from culture filtrates of a *C. fulvum* Avr9-overproducing transformant (Van den Ackerveken et al., 1993b), or chemically synthesised and folded *in vitro* (Van den Hooven et al., 1999). Plants that responded with an HR within four days upon injection of Avr4 or Avr9 were selected (one or two per accession). Injections were repeated at least three times to confirm the observed HR. For the initial screens, the FLAG-Avr4-B_{20} and *C. fulvum*-derived Avr9 were used. For subsequent injections, the wild type Avr4 and chemically synthesised Avr9 were used.

***Hcr9* amplification and cloning**

All DNA manipulations were performed using standard protocols (Sambrook and Russell, 2001). Restriction enzymes and T4 ligase were obtained from Promega (Madison, WI). Primers were synthesised by Sigma-Genosys (Cambridge, UK). To amplify as many *Hcr9*s per genotype as possible, primers were designed that anneal at the start and stop codon of all published *Hcr9*s and three Cf-Ecp2 *Orion Hcr9*s (De Kock et al., unpublished data; Parniske et al., 1997; Parniske and Jones, 1999; chapter 2). Several mixes of different primers in different concentrations were tested for amplification of *Hcr9*s from several *Lycopersicon* genotypes to give an optimal yield of PCR products. The mix that was used for PCR contained degenerate forward and reverse primers with 5' terminal *Nco*I and *Pst*I cloning sites, respectively, for subsequent cloning of the PCR product. Both forward (ATG) (Figure 3A) and reverse (TAG) primers end at three nucleotides that encode a conserved methionine residue, ensuring that the three terminal nucleotides are invariant. The following primers were used (in 5' to 3' direction, restriction sites underlined, start and stop codons in bold): ATG-N1: tttttt<u>cc**atg**g</u>rttgtgtaaaacttrtrttttratg, ATG-N2: tttttt<u>cc**atg**g</u>rttgtktaraacttrtattccttatg, ATG-N3: tttttt<u>cc**atg**g</u>rttgtttagaacttrtrttttratg, ATG-N4: tttttt<u>cc**atg**g</u>gttacgtramacttgtdttttthatg, TAG-P1: tttttt<u>ctgcag**cta**</u>atatctttttcttgtgctttttcat and TAG-P2: tttttt<u>ctgcag**yta**</u>ayatctttyctygtgyytttcat. Primers were used in the following ratios: ATG-N1:N2:N3:N4, 4:1:1:1; TAG-P1:P2, 2:1; and at a total concentration in the PCR mix of 28 ng/µl for the ATG-mix and 24 ng/µl for the TAG-mix. PCRs were performed on genomic tomato DNA (isolated as described by Van der Beek et al., 1992) using the Expand High Fidelity PCR System according to the manufacturers instructions (Roche, Basel, Switzerland), with an annealing temperature of 58°C. DNA fragments of the expected sizes (2.4-2.6 kb, Figures 3B and 3C) were excised from gel and purified using the GFX™ PCR DNA and gel band purification kit (Amersham-Pharmacia). Purified PCR products were digested with *Nco*I and *Pst*I and ligated into the *Nco*I and *Pst*I sites of pRH80 (Figure 3D) (Van der Hoorn et al., 2000). Individual clones containing a 35S promoter-*Hcr9*-PI-II terminator cassette were subsequently digested with *Xba*I and *Kpn*I (Figure 3E) and the fragments were cloned into the binary expression vector pMOG800 (Honée et al., 1998).

Agroinfiltration to select *Hcr9*s active in Avr4 or Avr9 recognition

Individual pMOG800 clones containing a single *Hcr9* amplified from an Avr4- orAvr9-responsive *Lycopersicon* plant were transformed to *A. tumefaciens* GV3101. These constructs were subsequently tested for their ability to confer Avr4 or Avr9 responsiveness using agroinfiltration in *Nicotiana tabacum* as described before, including the 35S-overexpression constructs pAvr4, pAvr9, pCf-4 and pCf-9 (Van der Hoorn et al., 2000). In a first screen for *Hcr9*s active in Avr4 or Avr9 recognition, *A. tumefaciens* colonies containing a single *Hcr9* were grown overnight in 3 ml cultures containing 2mM acetosyringone. The bacteria were pelleted and resuspended in MMA and used for agroinfiltration in leaves of *N. tabacum* cv Petite Havana SR1 (Van der Hoorn et al., 2000). *Hcr9*s derived from Avr4-responsive plants were tested for Avr4 recognition by co-infiltration with pAvr4, and *Hcr9*s derived from Avr9-responsive plants for Avr9 recognition by co-infiltration with pAvr9. For confirmation of the activity of single *Hcr9*s and for a reproducible comparison of their relative activity, clones were grown in 100 ml cultures as described in Van der Hoorn et al. (2000). Relative activities of *Hcr9*s mediating Avr4 or Avr9 recognition were determined in agroinfiltration dilution series (Van der Hoorn et al., 2001a).

Sequence analysis

pMOG800 clones active in Avr4 or Avr9 recognition were selected. pMOG800 is a low copy plasmid, which resulted in low quality sequence data. Therefore, the corresponding high copy pRH80 clone was sequenced double-stranded. Sequencing was carried out by BaseClear (Leiden, The Netherlands) with the following primers (given in 5' to 3' direction, length of the sequence run in brackets): pWLSF4 (800): cctctatataaggaagttcatttc, SF1 (800): cttaagaacttgacccaattaa, SF2 (800): tataccttcctggatattc, SF3 (500): gatcaaataagttgcatggtc, SF4 (500) cttggaaggtcatatacc, pWLSR (800): aacacaactttgatgcccac, SR1 (800):

ctcttaattgggtcaagttct, SR2 (800): gaatatccaggaaggtata, SR3 (800): ccatgcaacttattttgatctc, SR4 (500): ggtatatgaccttccaag. Sequences were analysed using Lasergene software programs (DNASTAR, Madison, WI). To verify that the obtained sequences did not contain PCR-induced mutations, at least one or two additional, independently isolated, active clones were sequenced. In the case of *Hcr9-Avr4-chl1*, all five sequenced clones were different from the wild type (consensus) sequence (all contained one or two independent mutations), in the case of *Hcr9-Avr4-per1*, four isolated clones differed from the wild type (consensus) sequence. Therefore, to test the activity of these *Hcr9-Avr4*s, clones that contained only one silent mutation were used. The sequences of the *Hcr9-Avr4*s and *Hcr9-Avr9*s reported in this study have been deposited with GenBank under accession numbers AY634610-AY634617. The sequence of *Hcr9-Avr4-hir1* was preciously submitted as *Cf-4* (Thomas et al., 1997).

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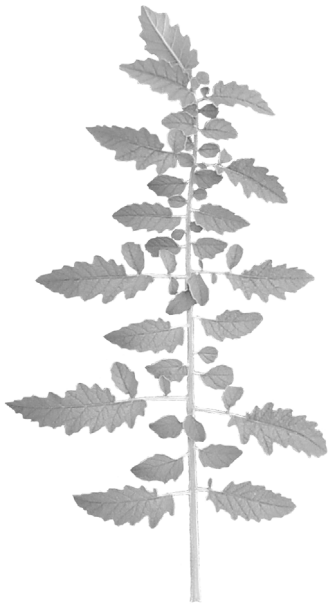
Chapter 5

Gene shuffling-generated and natural variants of the tomato resistance gene *Cf-9* exhibit different auto-necrosis-inducing activities in *Nicotiana* species



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Abstract

Tomato *Cf* genes encode membrane-bound proteins with extracellular leucine-rich repeats, and confer resistance to the fungal tomato pathogen *Cladosporium fulvum* by mounting defence responses that include an elicitor-induced hypersensitive response (HR). Several *Cf* genes, including *Cf-4* and *Cf-9*, are members of the highly homologous *Hcr9* (Homologues of *C. fulvum* resistance gene *Cf-9*) gene family. *Hcr9*s evolve mainly by sequence exchange between homologues, by which novel *Cf* genes may be generated. To mimic this aspect of natural evolution, we generated multi-chimeric *Hcr9*s *in vitro* by gene shuffling. The shufflants were tested for novel specificities by transient expression in *Nicotiana benthamiana*. Many shufflants induced an HR in the absence of fungal elicitors, and were designated auto-activators. We also identified two natural *Hcr9* auto-activators in the wild tomato species *Lycopersicon peruvianum*, which induced an HR upon expression in *N. benthamiana*. The *Hcr9* auto-activators described here represent the first auto-activators belonging to the *Cf*-like structural class of resistance genes. The *Hcr9* auto-activators exhibited different auto-necrosis-inducing activities in five selected *Nicotiana* species, and were shown to function in the same signalling pathway as *Cf-9*. Two models are proposed that explain the elicitor-independence of the auto-activators. In the 'receptor/elicitor' model, the auto-activators mediate recognition of endogenous *Nicotiana* factors that act as elicitors. In the 'polymorphic component' model, the different HR-inducing activities of the auto-activators are the result of (a) polymorphic component(s) of the Hcr9 signal transduction cascade in *Nicotiana*. The potential use of the auto-activators in unravelling the mechanisms of *Cf* gene-mediated resistance is discussed.

Race-specific pathogen resistance in plants is governed by disease resistance (*R*) genes, and usually complies with the gene-for-gene model (Flor, 1946). This model states that for a given plant-pathogen relationship, each *R* gene in the plant corresponds to an avirulence (*Avr*) gene in the pathogen. Upon *R* gene-mediated recognition of a cognate Avr elicitor protein, the plant mounts various defence responses that often includes a hypersensitive response (HR), and thereby restricts pathogen growth (Hammond-Kosack and Jones, 1996). Many *R* genes have been cloned over the last decade, and five main structural classes have been described (Martin et al., 2003; Nimchuck et al., 2003). Overexpression of *R* genes may

lead to induction of plant defence responses (Oldroyd and Staskawicz, 1998; Tang et al., 1999; Tao et al., 2000), whereas for some *R* genes, whose products belong to different structural classes, alleles that induce an elicitor-independent HR are known (Bendahmane et al., 2002; Chang et al., 2002; Hu et al., 1996; Hwang et al., 2000; Rathjen et al., 1999). Current research is focused on identifying domains that determine specificity within R proteins and signalling components of *R* gene mediated disease resistance.

The *Cladosporium fulvum*-tomato pathosystem follows the gene-for-gene relationship and is well suited to study plant disease resistance gene evolution and signalling (Joosten and De Wit 1999; Rivas and Thomas 2002). *C. fulvum* is a biotrophic fungus that colonises the intercellular spaces of the tomato leaf. Upon infection, *C. fulvum* secretes numerous small proteins, including the Avr and Ecp proteins that act as elicitors of the HR in certain tomato genotypes (Laugé et al., 2000). Recognition of these elicitors is mediated by *Cf* genes, which encode membrane-bound glycoproteins with a stretch of extracellular leucine-rich repeats (LRRs) and a short cytoplasmic domain that lacks an obvious signalling domain. Their localisation and structure is consistent with the proposed extracellular signal perception (Jones and Jones, 1997; Joosten and De Wit, 1999). The *Cf-4*, *Cf-4E* and *Cf-9* genes are all members of the *Hcr9* (Homologues of *Cladosporium fulvum* resistance gene *Cf-9*) family (Jones et al., 1994; Parniske et al., 1997; Takken et al., 1999; Thomas et al., 1997), whereas the *Cf-2* and *Cf-5* genes belong to the *Hcr2* subgroup (Dixon et al., 1996, 1998). Several studies have addressed the evolution of *Hcr9* gene clusters (Parniske et al., 1997, 1999; Wulff et al., 2004; chapter 3). It was shown that *Hcr9*s are composed of a patchwork of sequences, which has been generated by sequence exchange between individual homologues. Intra- and intergenic recombination, gene conversion, point mutation, duplication and translocation have all been implicated in the generation of *Hcr9* diversity.

Most *Hcr9*s encode 27 LRRs, however, exceptions include *Cf-4* and other *Hcr9*s that mediate Avr4 recognition (Parniske et al., 1997, 1999; chapter 4). The first 17 LRRs are highly variable, whereas the C-terminus of Hcr9 proteins is conserved. For example, the Cf-4 and Cf-9 proteins are identical from LRR 18 (of Cf-9) to their C-terminal ends, although they mediate recognition of two distinct elicitors with no sequence homology. This suggests that the specificity for elicitors is located in the N-terminus, and that the C-terminus has a conserved signalling function. Experiments in which Cf-4 and Cf-9 sequences were exchanged have identified specificity determinants in both proteins and showed that a large amino acid variation in Hcr9 proteins is allowed without loss of function (Van der Hoorn et al., 2001a; Wulff et al., 2001). This was further supported by the discovery of the *9DC* gene, which has the same specificity and activity in conferring Avr9 responsiveness as *Cf-9*, although the 9DC protein differs by 61 amino acids from Cf-9 (chapter 2).

Early *Cf*-mediated Avr-induced responses in tobacco cell cultures include the formation of active oxygen species, K^+ effluxes, medium alkalisation and an oxidative burst (Blatt et al., 1999; Piedras et al., 1998; De Jong et al., 2000). Moreover, two MAP kinases and a calcium-dependent protein kinase were identified that are rapidly activated upon Avr9

treatment (Romeis et al., 1999, 2000, 2001). Furthermore, *Cf* gene signalling was shown to depend on the ubiquitin ligase-associated protein SGT1 (Peart et al., 2002), and salicylic acid (SA) is required for the induction of the HR, but not for *C. fulvum* resistance (Brading et al., 2000). Recently, a Cf-9-interacting thioredoxin (CITRX) was identified as a negative regulator of Cf-9, but not of Cf-2 (Rivas et al., 2004).

Despite the present knowledge of *Cf*-mediated responses upon elicitation, little is known about how Cf proteins initiate downstream signalling events that lead to HR. Cf proteins are thought to act in complexes (Joosten and De Wit, 1999; Rivas and Thomas, 2002). Cf-4 and Cf-9 mediated signalling was shown to be temperature sensitive, possibly due to hampered formation of Cf-complexes (De Jong et al., 2002). However, no such complexes have been identified yet (Rivas et al., 2002a, 2002b; Van der Hoorn et al., 2003). A high-affinity binding site (HABS) for Avr9 has been identified in plasma membranes of Avr9-responsive and Avr9-non-responsive tomato plants, and also in several other Solanaceous species (Kooman-Gersmann et al., 1996). Moreover, no evidence for a direct interaction between Avr9 and Cf-9 was found (Luderer et al., 2000), suggesting an indirect interaction between Cf-9 and Avr9. For Avr4, no HABS could be detected in plasma membranes of tomato plants (Westerink et al., 2002), indicating an indirect perception of Avr4. Finally, Cf-2 mediated recognition of Avr2 requires the cysteine protease Rcr3 (Dixon et al., 2000; Krüger et al., 2002). As perception of Avr proteins mediated by Cf proteins appears to be indirect, Cf proteins may act as 'guards' of virulence targets of the Avr proteins (Van der Biezen and Jones, 1998; Van der Hoorn et al., 2002).

Gene shuffling provides a powerful tool to generate recombinant *Hcr9*s (Wulff et al., 2001), and can be used to mimic the generation of novel specificities of *Hcr9*s by natural evolution. We set out to identify novel specificities among gene shuffling-generated *Hcr9*s. Identification of novel specificities would not only provide experimental evidence for the versatile nature of *Hcr9*s in conferring elicitor recognition, but could also yield insight into specificity determinants within the Hcr9s and provide clues for common and unique signalling domains. *Cf-9* was previously shown to function in tobacco (Hammond-Kosack et al., 1998), and agroinfiltration in tobacco (*Nicotiana*) species is a well-established tool for testing the activity of *Hcr9*s (Thomas et al., 2000; Van der Hoorn et al., 2000, 2001a; Wulff et al., 2001; chapters 2, 3 and 4). Therefore, agroinfiltration was used to screen for *Hcr9* shufflants with novel specificities. Initially, we focussed on the identification of shufflants that could confer responsiveness to the *C. fulvum* elicitor Ecp1. However, shufflants that induced an HR in *N. benthamiana* leaves in the presence of Ecp1, induced the same response in the absence of Ecp1. We named these genes 'auto-activators', as they induce an elicitor-independent HR. Independently, two *Hcr9*s were recovered from the wild tomato species *L. peruvianum* that exhibit a similar auto-necrosis-inducing activity upon agroinfiltration in *N. benthamiana*. Some of the auto-activators appear to have different specificities as they exhibit different patterns of HR-inducing activities in five selected *Nicotiana* species. We further show that the auto-activators signal through the *Cf-9* pathway. Possible models that

may explain the auto-activation phenotype and the relevance of auto-activators for further studies are discussed.

Results

Construction of an *Hcr9* shuffled library

Comparison of natural *Hcr9*s has revealed that these genes are composed of patchwork sequences, suggesting that diversity in this gene family is mainly generated by inter- and intragenic recombination and gene conversion (Parniske et al., 1997; Parniske and Jones;; chapters 2 and 3). This process of *Hcr9*-recombination can be accelerated *in vitro* by using gene shuffling, a technique in which a pool of related genes are fragmented, and subsequently assembled into full length genes by a self-priming PCR process yielding a library of chimeras (Stemmer, 1994). We set out to shuffle multiple *Hcr9* sequences with a view to generate a large library of chimeras with a potential spectrum of novel recognition specificities.

Plasmid or PCR-derived fragments, which encode (parts of) the variable N-terminus of the Hcr9s, extending from the ATG start to the region encoding LRR21, of the *ΨCf-0* (the *Ψ* prefix indicates pseudo genes), *Cf-4*, *4E*, *9B*, *Cf-9, 9D*, *ΨSCB*, *ΨNLA* and *NLC Hcr9*s (Jones et al., 1994; Parniske et al., 1997; Parniske and Jones, 1999; Thomas et al., 1997) were used as input for the generation of a Hcr9 shuffled library. These fragments were digested with different combinations of frequently cutting restriction enzymes to yield fragments with an average size of 90 bp (Figure 1), which were reassembled into chimeras by gene shuffling (Stemmer, 1994). Shuffled sequences were cloned in the binary expression vector pSLJ12904 between the 35S promoter and the 3' half of *Cf-9* (Wulff et al., 2001). The resulting constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101. The efficiency of the cloning was assessed by PCR on 32 random clones. Thirty clones (94%) contained inserts, of which five clones (17%) encoded Hcr9s with 25 LRRs, and 25 clones (83%) encoded Hcr9s with 27 LRRs. The efficiency of the shuffling was analysed by sequencing of six randomly picked clones (clones 12.1.1 to 12.2.6, Figure 2). The average number of crossovers per clone detectable at the nucleotide level was 18, and 13.2 at the amino acid level. The crossovers appeared to be evenly distributed (Figure 2). No shuffling-associated point mutations were detected in the six clones, so the point mutation rate was <1 per 10.5 kb.

Screening of the *Hcr9* shuffled library for novel specificities

To identify novel recognition specificities among the diverse Hcr9s of the *Hcr9* shuffled library, we first set out to identify clones with the capacity to confer Ecp1 recognition *in planta*. Ecp1 is one of the *C. fulvum* elicitor proteins which elicits an HR when expressed or infiltrated in the leaves of certain accessions of the wild tomato species *Lycopersicon pimpinellifolium* (Laugé et al., 2000).

Hcr9	Restriction enzymes used	Sites
9B	*Hinf*I, *Mnl*I, *Rsa*I	16
9B	*Dpn*I, *Mse*I	16
9D	*Alu*I, *Tsp*509I	27
Cf-9	*Cvi*JI*	37
Cf-9	*Acc*I, *Alu*I, *Bfa*I, *Sau*96I, *Sna*BI	23
Cf-9[Δ2LRR]	*Bsm*AI, *Mse*I, *Sty*I	3
Cf-4	*Hin*fI, Tsp509I	17
Cf-4	*Alu*I, *Xmn*I	12
4E	*Alu*I, *Nla*III	17
ΨCf-0	*Bsp*HI, *Bst*XI, *Eco*RI, *Hae*III, *Ssp*I	7
ΨSCB	*Ava*I, *Bfa*I, *Dde*I, *Hae*III, *Sna*BI,	7
ΨNLA	*Hae*III, *Ssp*I, *Xmn*I	4
NLC	*Acc*I, *Bfa*I, *Eco*RI, *Pvu*II	5

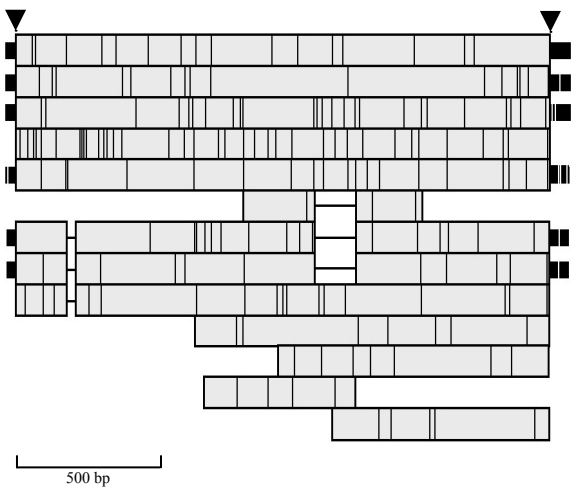


Figure 1. Restriction enzyme-mediated fragmentation of DNA sequences used for generation of the shuffled *Hcr9* library.

Hcr9 DNA sequences used for shuffling are shown by grey rectangles and pBluescript polylinker anchors used to rescue full length shuffled clones by PCR (Wulff et al., 2001) are indicated by black rectangles. The two arrow heads indicate the unique restriction sites that were used for cloning shuffled products in the binary expression vector (a *Cla*I site at the ATG start codon and a *Hind*III site in the region encoding LRR 21).The number of restriction sites in each *Hcr9* template is indicated in the column, and the position of each site is shown by thin vertical lines.

In a pilot experiment, 720 *Hcr9* shufflants were screened by *Agrobacterium*-mediated transient assays (Thomas et al., 2000; Van der Hoorn et al., 2000). *Agrobacterium* cultures containing individual shufflants were mixed with an *Agrobacterium* culture containing a T-DNA construct encoding ECP1 fused to the tobacco PR1A signal peptide under control of the constitutive 35-S promoter (C. Mills and C. Thomas, unpublished data), and infiltrated into mature leaves of *N. benthamiana* plants. Fifty-three of these shufflants (7.4%) were found to induce an HR. However, upon rescreening, all of these 53 clones were found to induce the same degree of HR in the absence of Ecp1 (data not shown). The high background of clones with an auto-necrosis-inducing phenotype in *N. benthamiana* indicated that this species was not suitable for this screen. *N. tabacum* was also unsuitable, as screening of 100 random clones yielded a similarly high percentage of auto necrosis-inducing shufflants (designated auto-activators). Due to the high background of these auto-activators, the screen for Ecp1-recognising clones was abandoned.

Surprisingly, the HR-inducing activity of some auto-activators was different between *N. benthamiana* and *N. tabacum*. This was initially established for two auto-activators, named *Auto 1* and *Auto 2*. When *Auto 1* is expressed in *N. tabacum*, the whole infiltrated leaf area becomes necrotic, whereas it induces no or hardly any detectable (threshold) HR in *N. benthamiana* leaves (Figure 3). *Auto 2* on the other hand, induces a strong HR in *N. benthamiana*, but only an intermediate HR in *N. tabacum* (Figure 3).

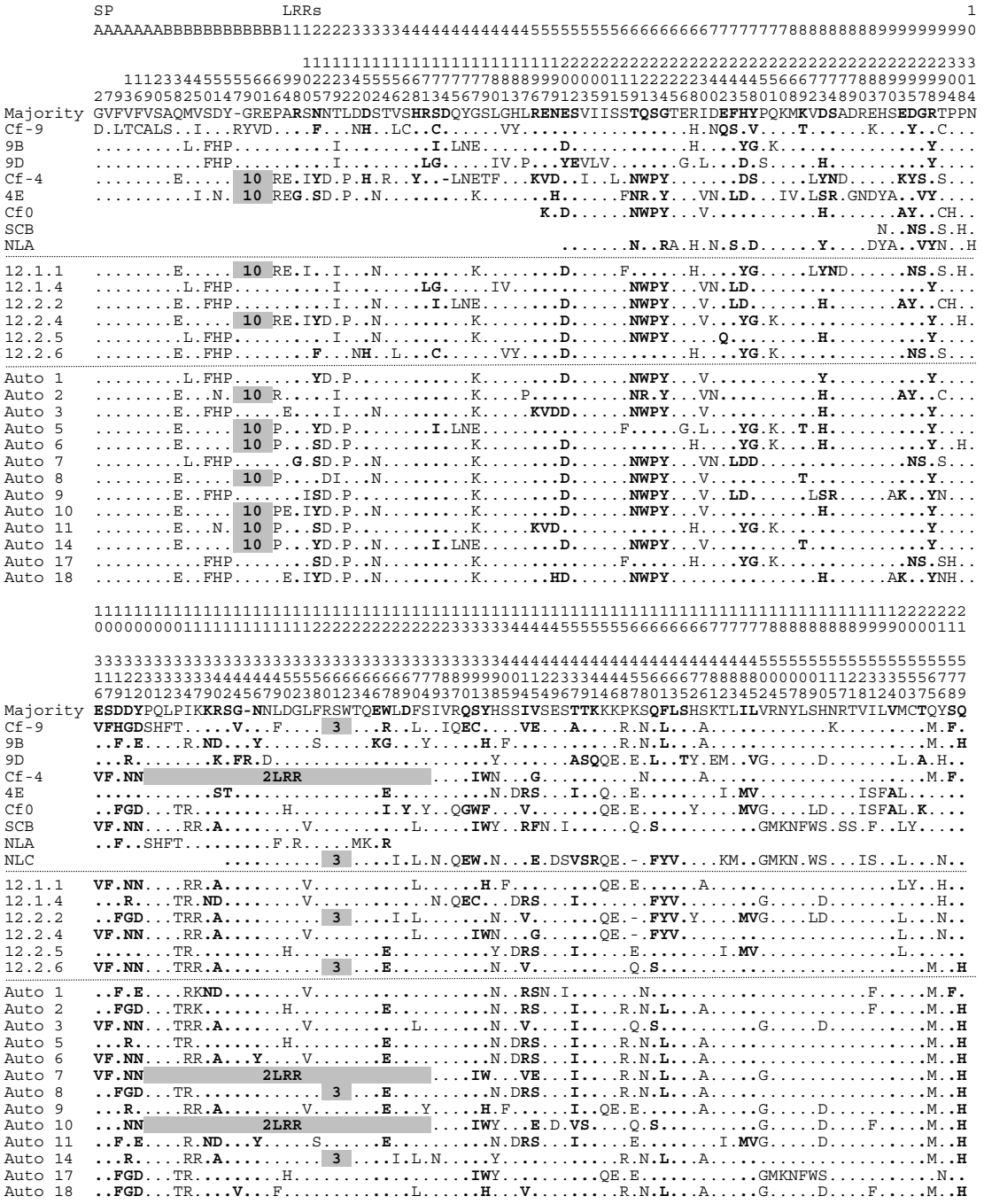


Figure 2. Amino acid sequence alignment of shuffled Hcr9 proteins.
Only polymorphic amino acids are shown in the alignment. Their location within domain A (A), the putative signal peptide (SP), domain B (B), and LRRs (1 to 21) are indicated in the upper panel. Vertical numbers refer to the amino acid position in the alignment. Amino acids that correspond to putative solvent-exposed residues of the LRR β-strand/β-turn motif are indicated in bold lettering. Majority indicates the amino acid found in the majority of the input Hcr9s at a certain position. Single amino acid deletions are indicated by dashes; grey boxes indicate deletions of three or ten adjacent amino acids or of two complete LRRs. The Hcr9 protein fragments used in the shuffling (top) are separated by dotted lines from six randomly picked shuffled clones (middle) and the auto-activators (bottom). Of the pseudo (Ψ) genes *ΨCf-0*, *ΨSCB* and *ΨNLA* only fragments that do not contain nonsense mutations were included in the shuffling. The average minimum number of shuffling-generated crossovers detected at the amino acid level was 13.2 in the randomly picked clones, and 13.7 in the auto-activators.

Different patterns of HR-inducing activities of shuffled auto-activators

To test whether the different patterns of HR-inducing activities in *N. benthamiana* and *N. tabacum* of *Auto 1* and *Auto 2* are a common phenomenon, the screen was enlarged by including more *Hcr9* shufflants and more *Nicotiana* species. *N. tabacum*, *N. benthamiana*, *N. knightiana*, *N. undulata* and *N. rustica* (hereafter referred to as *Nicotiana* testers) were chosen for further analysis of the auto-activators out of a total of twenty screened *Nicotiana* species. The leaves of the *Nicotiana* testers were easy to infiltrate, revealed little or no aspecific necrosis associated with the expression of a 35S:*GFP* T-DNA construct, and the accumulation of GFP was high as judged by fluorescence under UV light (data not shown). Expression of the full-length *Cf-4* and *Cf-9* clones in the *Nicotiana* testers induced an HR only in the presence of the matching Avr4 and Avr9 elicitors, respectively (Table 1), indicating that the Cf-4 and Cf-9 pathways are intact in these species.

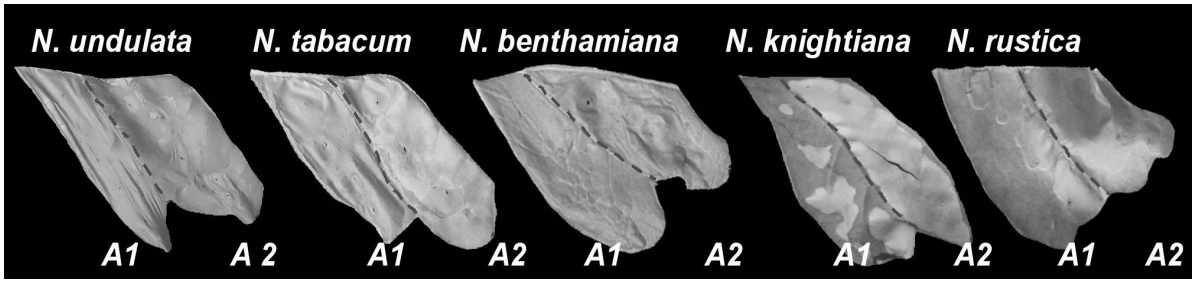


Figure 3. HR-inducing activities of *Auto 1* and *Auto 2* in the *Nicotiana* testers.
HR was induced by *Agrobacterium*-mediated transient expression of *Auto 1* (*A1*, left leaf sections) and *Auto 2* (*A2*, right leaf sections) in the *Nicotiana* testers (see also Table 1). Only the infiltrated leaf sections are shown.

Auto 1, *Auto 2* and an additional 11 *Hcr9* shufflants which exhibited some degree of HR-inducing activity in *N. benthamiana* in the preliminary screen, were transiently expressed in the *Nicotiana* testers. The six randomly picked shufflants (Figure 2) that were used to assess the shuffling efficiency were also screened. The shufflants could be grouped into five distinct classes, based on their different HR-inducing activities. One of these classes, which included all randomly picked shufflants, was defined by inducing no necrosis in any of the

Table 1. *Hcr9* auto-activators exhibit different HR-inducing activities in different *Nicotiana* species.

	N. benthamiana	*N. knightiana*	*N. rustica*	*N. tabacum*	*N. undulata*	Class
Auto 1	±	+	++	++++	++++	1
Auto 2	+++	++++	++++	++	++	2
Auto 3	±	++	++	±	±	3
Auto 5	+	++	++	-	-	3
Auto 6	-	+	+	-	-	3
Auto 7	±	++	+++	±	-	3
Auto 8	++	+	++	-	-	3
Auto 9	+	+++	+++	+	+	3
Auto 10	±	+++	+++	-	+	3
Auto 11	+	++	+	±	-	3
Auto 14	-	+	±	-	-	3
Auto 17	+	+++	++	++	±	4
Auto 18	++	+++	++	++	±	4
peru 1	++	+++	++	++++	+++	5
peru 2	+++	++++	+++	++++	++++	5
12.1.1	-	-	-	-	-	0
12.1.4	-	-	-	-	-	0
12.2.2	-	-	-	-	-	0
12.2.4	-	-	-	-	-	0
12.2.5	-	-	-	-	-	0
12.2.6	-	-	-	-	-	0
ΨCf-0:Cf-9	-	-	-	-	-	0
Cf-4	-	-	-	-	-	0
Cf-9	-	-	-	-	-	0
9B	±	++	+	-	++	6
9D	-	-	-	-	-	0
NLC	-	-	-	-	-	0
4E	-	-	-	-	-	0
Cf-4 + Avr4	++++	++++	++++	++++	++++	
Cf-9 + Avr9	+++	++++	++++	++++	++++	

++++ = confluent necrosis in the whole infiltrated leaf sector; +++ = necrosis in ~75% of the infiltrated leaf sector; ++ = ~50% necrosis; + = ~25% necrosis; ± = threshold activity; - = no necrosis.
Infiltrations were repeated in different experiments at least 5 times per construct in each species, and the relative HR-inducing activities of *Auto 1* to *Auto 18* were compared side-by-side in the same leaves, always with the controls *Auto 1*, *Auto 2* and *Cf-9*. *Autos* are classified (classes 0-6) according to their pattern of auto-necrosis-inducing activities in the different *Nicotiana* species.

Nicotiana testers (class 0, Table 1). *Auto 1* and *Auto 2* each fell into a distinct class (classes 1 and 2, Table 1). *Auto 1* induced a strong, confluent HR (necrosis throughout the complete infiltrated leaf area) in *N. undulata* and *N. tabacum*, whereas the activity of *Auto 2* was intermediate (about 50% necrosis) in these species. On the other hand, *Auto 1* induced a weak (up to 25%) or threshold HR in *N. benthamiana*, *N. knightiana* and *N. rustica*, whereas the activity of *Auto 2* was relatively strong (75%-100% necrosis) in these species (Figure 3, Table 1). A class containing nine shufflants could be loosely defined as inducing a weak or threshold HR in *N. undulata*, *N. tabacum*, and *N. benthamiana*, and inducing intermediate levels of HR in *N. knightiana* and *N. rustica* (class 3, Table 1). Another class, containing

Auto 17 and *Auto 18*, was defined by inducing a threshold HR in *N. undulata*, but intermediate levels of HR in *N. tabacum*, *N. benthamiana*, *N. knightiana* and *N. rustica* (class 4, Table 1).

Full-length binary constructs were made for *4E*, *9B*, *9D* and *NLC* to test whether novel auto-activator specificities had been generated by the shuffling, or whether old specificities encoded by the input *Hcr9* fragments had been reshuffled and reselected. No full-length constructs were made for the pseudo genes *ΨCf-0*, *ΨSCB* and *ΨNLA*, as these are predicted to be non-functional. In *ΨCf-0* this is due to a deletion of 60 bp encoding part of the signal peptide (Parniske et al., 1997), and in *ΨSCB* and *ΨNLA* nonsense mutations lead to predicted truncated proteins of 194 and 173 amino acids, respectively (Parniske et al., 1999). Therefore, as expected, a *ΨCf-0* construct consisting of the 5' half of *ΨCf-0* fused to the 3' half of *Cf-9* in the region encoding LRR 21 (named *ΨCf-0*:*Cf-9)* did not induce HR in any of the *Nicotiana* testers (Table 1). Full-length constructs of *4E*, *9D* and *NLC* did not induce any HR in the *Nicotiana* testers either. Expression of *9B*, however, induced an intermediate HR in *N. undulata* and *N. knightiana*, but no or only a very weak HR in the other species (Table 1). Thus, *9B* represented a class of auto-activators not found among the shufflants (class 6, Table 1). Therefore, the identified classes of HR-inducing shufflants represent novel auto-activator specificities generated by *Hcr9* gene shuffling and selection.

Sequence analysis of the shuffled auto-activators

The variation in the shuffled auto-activators may in part have been generated by PCR-induced point mutations. To determine the relative contribution of recombination and point mutation to the generation of the auto-activator specificities, all 13 shuffling generated auto-activators were sequenced. No novel mutations were detected at the DNA level. Furthermore, no new amino acid sequences were generated by shuffling mediated intra-codon recombination. Therefore, the novel auto-activator specificities were generated by recombination only. The average detectable number of recombination events in each auto-activator was 13.7 at the amino acid level (Figure 2).

The auto-activators clearly exhibit different activities of HR-inducing activity in the *Nicotiana* testers, although their sequences differ significantly (Figure 2). For instance, auto-activators with or without the 10 amino acid deletion in the B-domain have been identified, as well as auto-activators with or without the two LRR deletion. However, phylogenetic analysis and visual inspection of the auto-activator sequences did not identify any sequences that could be correlated with the different auto-activator classes (data not shown).

Natural auto-activators of *L. peruvianum*

To study if natural auto-activators, rather than only artificially generated, also occur, we amplified many different *Hcr9*s from a selection of tomato plants representing all eight wild species (Warnock et al., 1988), using a degenerate primer mix as described previously

(chapter 4). These *Hcr9*s were cloned in the binary expression vector pMOG800 (Honée et al., 1998) under control of the 35S promoter as described previously (chapters 2 and 4). To identify potential auto-activators, these *Hcr9*s were transiently expressed in *N. benthamiana* by agroinfiltration, and two *Hcr9*s of *L. peruvianum* were identified that induced an elicitor-independent HR. The HR-inducing activities of these two additional auto-activators, named *peru 1* (isolated from *L. peruvianum* LA0441) and *peru 2* (isolated from *L. peruvianum* LA1984) were also determined in the other four *Nicotiana* testers. The activities of *peru 1* and *peru 2* were similar to that of *Auto 2*, however, *Auto 2* induces an intermediate HR in *N. undulata* and *N. tabacum*, and the HR induced by *peru 1* and *peru 2* was significantly stronger in these species (Table 1). Therefore, *peru 1* and *peru 2* were grouped in a new class (class 5, Table 1).

The nucleotide sequences of *peru 1* and *peru 2* resemble the patchwork sequences found in other *Hcr9*s (Parniske et al., 1997; Parniske and Jones, 1999). Both *peru 1* and *peru 2* encode proteins that contain 27 LRRs, and have a high amino acid identity with each other (95.2%) and with homologue 4B of the *Cf-4 Milky Way* cluster (95.4% and 95.6%, respectively) (Parniske et al., 1997). The identification of *peru 1* and *peru 2* demonstrates that naturally occurring *Hcr9s* can also act as auto-activators when transiently expressed in different *Nicotiana* species.

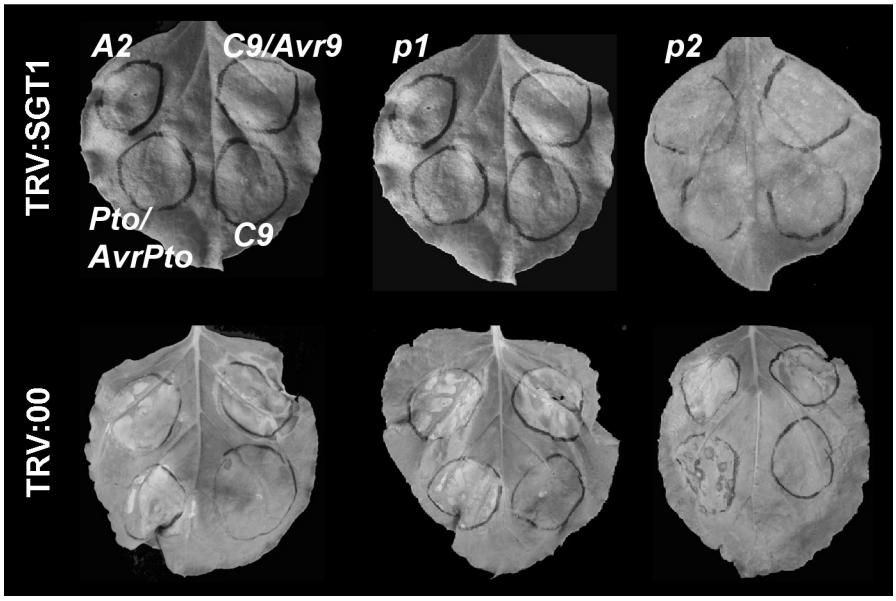
Auto-activators signal through the Cf-9 pathway

An important question is how auto-activators induce an elicitor-independent HR in *Nicotiana* species, and how they can be employed to help unravel the mechanism by which *Cf-4* and *Cf-9* mediate activation of defence and resistance responses. To help address these questions it is crucial to establish whether the auto-activators signal through the Cf-9 pathway. Several tools are available to study the Cf-9 signalling pathway in *N. benthamiana*. We focused on *Auto 2*, *peru 1* and *peru 2*, because these induce the strongest HR in *N. benthamiana* (see Table 1), and represent both artificial and natural auto-activators.

The ubiquitin ligase-associated protein SGT1 is required for Cf-4- and Cf-9-mediated HR, and plays a pivotal role in plant disease resistance mediated by structurally diverse *R* genes (Austin et al., 2002; Azevedo et al., 2002; Peart et al., 2002). We tested the requirement of SGT1 for the HR mediated by *Auto 2*, *peru 1* and *peru 2*, by Tobacco Rattle Virus (TRV) mediated silencing of *Sgt1* in *N. benthamiana* (Peart et al., 2002). The HR-inducing activity of these three auto-activators and the control, *Cf-9* with *Avr9*, was abolished in *Sgt1* silenced plants, but not in control plants infected with a TRV empty vector (Figure 4A, Table 2).

The plant defence-related signalling molecule SA is required for the HR mediated by *Cf-9* in tomato and tobacco (Brading et al., 2000). To determine whether *Auto 2*, *peru 1* and *peru 2* require SA for their induction of HR in *N. benthamiana*, the bacterial salicylate hydroxylase gene *nahG* was transformed into *N. benthamiana*. A NahG transgenic line with

A



B

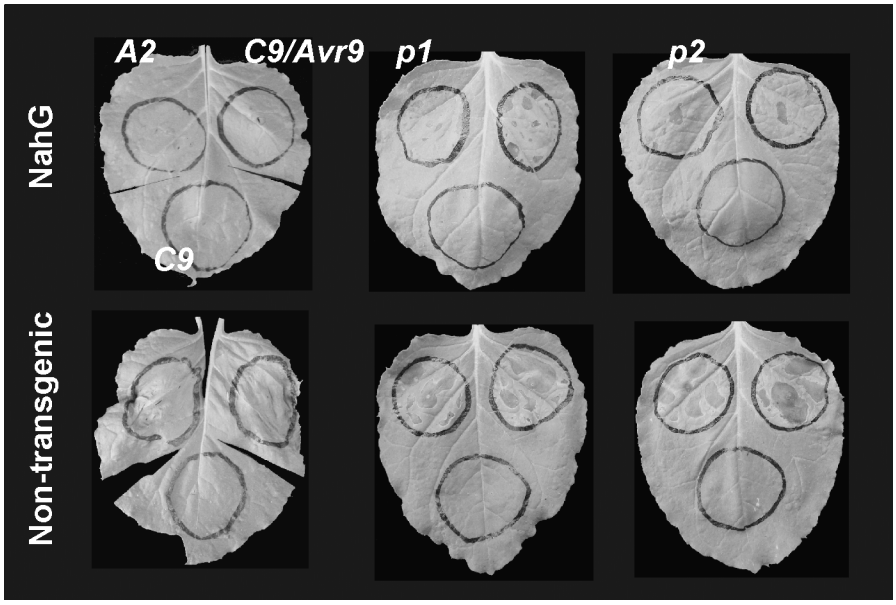


Figure 4. HR-inducing activities of selected auto-activators in TRV:SGT1 silenced, TRV:00 silenced, transgenic NahG and non-transgenic *N. benthamiana* plants.

HR was induced by *Agrobacterium*-mediated expression of *Auto 2* (*A2*), *peru 1* (*p1*), *peru 2* (*p2*) and the controls *Cf-9* with *Avr9* (*C9*/*Avr9*), *Cf-9* (*C9*) and *Pto*/*AvrPto*.

A. *Sgt1* is required for the function of *Auto 2*, *peru 1* and *peru 2*. *N. benthamiana* plants were silenced with a tobacco rattle virus (TRV) construct containing *Sgt1* (TRV:SGT1, top panel, Peart et al., 2002), or a TRV empty vector control (TRV:00, bottom panel) (see also Table 2).

B. Salicylic acid (SA) is required for the function of *Auto 2*, *peru 1* and *peru 2*. SA was depleted in transgenic NahG *N. benthamiana* plants (top panel) but not in the non-transgenic control plants (bottom panel) (see also Table 2).

undetectable levels of SA did not support the confluent development of HR normally mediated by *Auto 2*, *peru 1*, *peru 2* or the control, *Cf-9* with *Avr9* (Figure 4B, Table 2).

CITRX is a negative regulator of the Cf-9 protein, but not of the highly homologous Cf-2 protein (Rivas et al., 2004). *CITRX* silenced *N. benthamiana* plants show an accelerated *Cf-9/Avr9* mediated HR (Rivas et al., 2004). Conversely, when *CITRX* is overexpressed, the HR mediated by *Cf-9* is significantly attenuated. Similarly, CITRX was also found to be a negative regulator of the Cf-4-mediated HR (data not shown). Therefore, *CITRX* provides an excellent tool to address whether *Auto 2*, *peru 1* and *peru 2* signal specifically through the Cf-4 and Cf-9 pathway. When *Auto 2*, *peru 1* and *peru 2* were transiently expressed in *CITRX* silenced *N. benthamiana* plants, the HR was significantly enhanced compared to control plants silenced with an empty vector construct (Figure 5A, Table 2). Conversely, when a construct overexpressing *CITRX* was co-expressed with *Auto 2*, *peru 1* and *peru 2*, the HR was significantly attenuated (Figure 5B, Table 2).

The results with *Sgt1*, SA and especially *CITRX* demonstrate that the phenotypes of *Auto 2*, *peru 1* and *peru 2* are caused by deregulation of a common element of the Cf-4/Cf-9 signalling pathways, rather than a general protein toxicity effect associated with overexpression.

Auto 1 induces only a threshold HR in *N. benthamiana*. Surprisingly, transient expression of *Auto 1* in *CITRX* silenced *N. benthamiana* plants induced a full confluent HR, whereas the negative control *Cf-9* did not induce an HR (Figure 5A, Table 2). This indicates that *Auto 1* is a potential auto-activator in *N. benthamiana* that signals through the *Cf-4*/*Cf-9* pathway, and that removal of the negative regulator CITRX is enough to initiate signalling leading to a full HR.

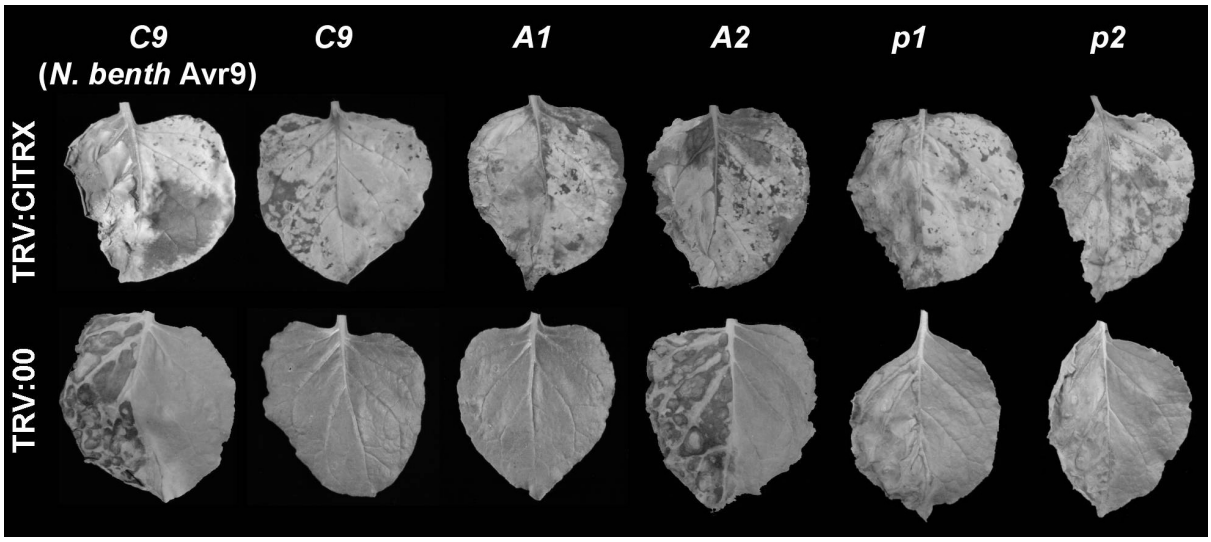
Discussion

Novel *Hcr9* auto-activator specificities generated by gene shuffling

By using members of the conserved *Hcr9* gene family as input for a gene shuffling experiment, a great variety of novel *Hcr9* shufflants was generated. Some of these shufflants encoded novel specificities and were found to act as auto-activators of HR in *Nicotiana* species. Comparison of the HR-inducing activities of these auto-activators with full length constructs of the input clones used for the shuffling, showed that at least four novel specificities had been generated (Table 1).

Previously, a screen of >700 *Cf-4/Cf-9*-shuffled clones did not lead to identification of any auto-activators in *N. benthamiana* or *N. tabacum* (Wulff et al., 2001; data not shown), which indicates that within *Cf-4* and *Cf-9* there is no or little potential for auto-activation of HR in these species. In contrast, a high proportion of auto-activators was recovered from the *Hcr9*-shuffled library used in this study.

A



B

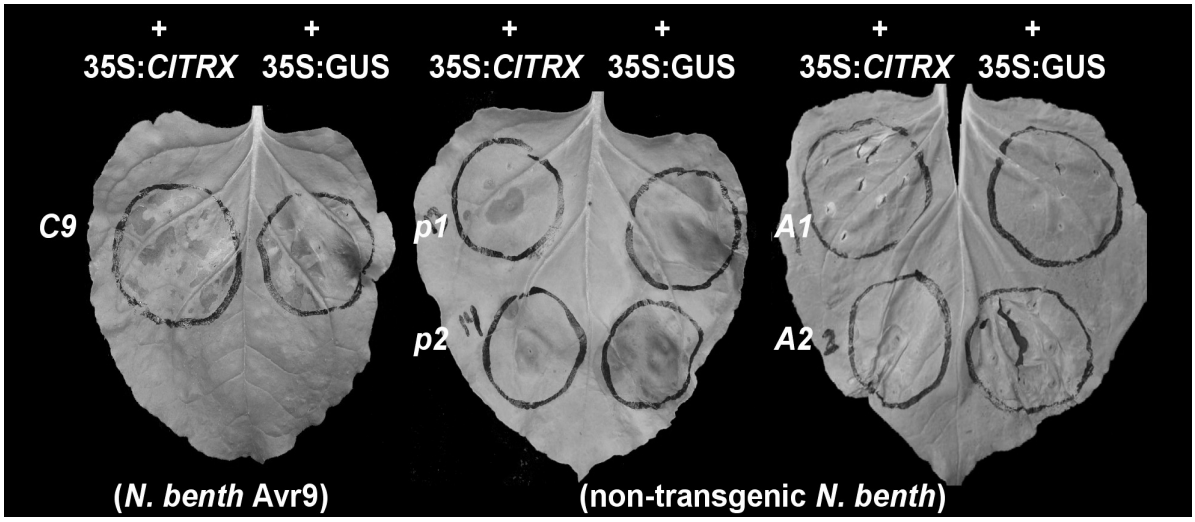


Figure 5. CITRX is a negative regulator of Auto 1, Auto 2, peru 1 and peru 2.
HR was induced by *Agrobacterium*-mediated expression of *Auto 1* (*A1*), *Auto 2* (*A2*), *peru 1* (*p1*) and *peru 2* (*p2*) and the control *Cf-9* (*C9*).
A. HR induced by selected auto-activators in TRV:*CITRX* silenced (top panel) and TRV:00 silenced *N. benthamiana* plants. CITRX is a negative regulator of Cf-9 (Rivas et al., 2004). Silencing of *CITRX* results in bleaching of the leaf, independent of infiltrations. The left side of the leaves was infiltrated with the different *Hcr9*s, whereas the right side was not infiltrated. See also Table 2.
B. HR induced by expression of *Cf-9* in *N. benthamiana* Avr9 (left leaf), by *peru 1* and *peru 2* in non-transgenic *N. benthamiana* (middle leaf), and by *Auto 1* and *Auto 2* in non-transgenic *N. benthamiana* (right leaf), is attenuated by *Agrobacterium*-mediated co-expression of *CITRX*, compared to co-expression of GUS (β-glucoronidase) (see also Table 2).

Some of the input *Hcr9* fragments were derived from pseudo genes of which expression would lead to predicted mutant proteins lacking the signal peptide (ΨCf-0), or prematurely truncated proteins (ΨSCB and ΨNLA). Several studies have found that truncated mutant proteins of Cf-9 lacking the TM domain and various numbers of LRRs are non-functional (Thomas et al., 2000; Van der Hoorn et al., 2003; Wulff et al., 2004). Therefore, the pseudo genes might contain latent auto-activator domains. Shuffling could have restored these domains leading to a functional auto-activator. Possibly, these pseudo genes were once *R* genes that, to some extent, constitutively activated defence responses. This would have imposed a fitness cost, and would therefore have been selected against in the absence of pathogen pressure (Tian et al., 2003), upon which these genes may have become pseudo genes. Alternatively, as pseudo genes are predicted to be non-functional, mutations that would normally lead to auto-activation of HR in tomato may have accumulated. Both the auto-activators and the non-active random clones share amino acids with the pseudo genes, and some contain amino acids only present in one of the input pseudo genes (Figure 2). Therefore, it would be useful to establish whether restoration of the *ΨCf-0*, *ΨSCB* and *ΨNLA* ORFs would result in *Hcr9* auto-activators. Should restoration of these ORFs indeed lead to auto-activation of HR in *Nicotiana*, these ORFs may be omitted from novel *Hcr9*-shuffled libraries.

Although the current *Hcr9*-shuffled library could not be used to identify *Hcr9*s that mediate novel recognition specificities for *C. fulvum* elicitors, the non-auto-activating shufflants may be tested for conferring recognition of Avr4 and/or Avr9. The sequences of such clones will contribute to studies on the plasticity of Avr4 and Avr9 recognition (Van der Hoorn et al., 2001a; Wulff et al., 2001).

Naturally occurring auto-activators

Peru 1 and *peru 2* were isolated from two *L. peruvianum* plants of different accessions. Although both genes exhibit a strong HR-inducing activity in the *Nicotiana* testers, no symptoms were observed in the *L. peruvianum* plants themselves (data not shown). This suggest that *peru 1* and *peru 2* do not act as auto-activators of HR under control of their native promoter in *L. peruvianum*. The *9B* gene from the *Cf-9* cluster (Parniske et al., 1997) was also found to induce HR in some of the *Nicotiana* testers. The intrinsic function of *peru 1* and *peru 2* is not known, but *9B* confers partial resistance to *C. fulvum* (Laugé et al., 1998a; Parniske et al., 1997; Panter et al., 2002). *Peru 1* and *peru 2* may also have a function in pathogen resistance, or may play a role in the generation of novel *Hcr9*s via sequence exchange with other *Hcr9*s (Parniske et al., 1997; Parniske and Jones, 1999; chapters 2 and 3). *Peru 1* and *peru 2*, or even *Hcr9*s in general, may also play a role in maintenance of distinct *Lycopersicon* species. Although some tomato species are compatible and can therefore be manually crossed, separate species are maintained in nature (Rick, 1979). Hybrids might suffer from auto-necrosis when an Hcr9 from one parental species recognises

an endogenous 'elicitor protein' from the other parental species, and will therefore be selected against. For instance, *Cf-2*-dependent auto-necrosis occurs in plants that carry the *Cf-2* gene from *L. pimpinellifolium* in combination with the *L. esculentum* allele of *Rcr3* (*Rcr3*^{esc}), but not in combination with the *L. pimpinellifolium* allele (*Rcr3*^{pim}) (Krüger et al., 2002).

Auto-activating alleles of other *R* genes

In this study, the first auto-activators of HR of the *Cf*-like type of *R* genes are described. Auto-activator alleles of several other types of *R* genes have been described previously. Alleles encoding the intracellular serine/threonine Pto with mutations in the P+1 loop of the kinase activation domain induce HR when expressed in *N. benthamiana* and tomato (Rathjen et al., 1999). Similarly, the *Pto* homologues *LpimPth3* and *LpimFen* induce cell death upon expression in *N. benthamiana*, whereas *LescFen* does not (Chang et al., 2002). Alleles of the NB-LRR gene *Rx* that carry point mutations in the NB and LRR regions also induced HR in *N. tabacum* (Bendahmane et al., 2002). In another NB-LRR family, four disease lesion mimic mutants were shown to result from mutations at the *Rp1* locus in maize (Hu et al., 1996), including a recombinant *Rp1* gene resulting from intragenic recombination in the LRR regions between two paralogs at the *Rp1-D* locus (Sun et al., 2001). Transient expression of domain swap alleles of the CC-NB-LRR gene *Mi-1.1* (created by exchanging parts of the LRR region with that of *Mi-1.2*) in *N. benthamiana* leaves resulted in localised cell death, whereas expression of *Mi-1.1* or *Mi-2.1* did not (Hwang et al., 2000).

The auto-activators described for other types of *R* genes are reminiscent of the *Hcr9* auto-activators identified in this study. A simple explanation for the mechanism of these *R*-gene auto-activators is that these mutants facilitate constitutive downstream signalling and the development of defence responses, including an HR, independent of the presence of a pathogen-derived elicitor molecule. However, all auto-activating alleles described above have signalling domains within the encoded proteins, whereas Hcr9s do not contain an obvious signalling domain. Furthermore, if the *Hcr9* auto-activators constitutively activate defence responses, it would be expected that they produce the same phenotype in all *Nicotiana* species tested, which is clearly not the case (Table 1).

Models to explain the different *Hcr9* auto-activator activities

The constitutive activation of various defence responses such as microscopic cell death, SA accumulation and/or PR gene transcript-accumulation has been observed when overexpressing *R* genes, including *Pto*, *Prf* and *Rps2* (Oldroyd and Staskawicz, 1998; Tang et al., 1999; Tao et al., 2000). This observation is explained by a model in which the *R* genes are proposed to have a low basal activity in the absence of their elicitors. By overexpression of the *R* gene the threshold activity required for induction of an HR is exceeded (Tao et al., 2000; Tang et al., 1999). In this study, all *Hcr9*s were overexpressed in transient assays under control of the 35S promoter. Although this overexpression does not lead to an HR with the controls *Cf-4* and *Cf-9* in the absence of their corresponding elicitors (Thomas et al., 2000;

Van der Hoorn et al., 2000, 2001a; Wulff et al., 2001; this study), it is possible that the auto-activation phenotype observed with the *Hcr9* auto-activators can be attributed, at least in part, to overexpression. Indeed, it was shown that a direct correlation exists between Cf-9 protein accumulation and HR-inducing activity in the presence of Avr9 (Van der Hoorn et al., 2003; Voinnet et al., 2003). However, this ‘overexpression' model fails to explain adequately why some auto-activators would be overexpressed in some *Nicotiana* species, but not in others, and therefore give rise to the different classes of HR-inducing activities (Table 1).

Two alternative models may explain the different HR-inducing activities of the *Hcr9* auto-activators (Table 1). First, in a simple 'receptor/elicitor' model each class of auto-activators mediates recognition of a different endogenous *Nicotiana* factor, which then act as an elicitor of HR. The presence, absence or relative concentration of these endogenous elicitors in different *Nicotiana* species determines whether an *Hcr9* acts as an auto-activator or not.

The second model is a variation of the 'receptor/elicitor' model. In this 'polymorphic component' model, the different HR-inducing activities of an *Hcr9* auto-activator in different *Nicotiana* species are the result of polymorphism in a *Nicotiana* component(s) involved in the signal transduction leading to HR. This polymorphism could result in a differential interaction of the Hcr9 auto-activator with such a component(s), leading to different HR-inducing activities in different *Nicotiana* species.

A precedent for the 'polymorphic component' model is provided by the tomato cysteine protease Rcr3, which is required for *Cf-2*-dependent disease resistance but not for *Cf-5* or *Cf-9* (Dixon et al., 2000; Krüger et al., 2002). When *Cf-2* is expressed in combination with the *L. esculentum* allele of *Rcr3*, but not with the *L. pimpinellifolium* allele, this results in necrosis in adult plants. Strikingly, $Rcr3^{esc}$ and $Rcr3^{pim}$ are 98% identical and differ only by six amino acids and a deletion of one amino acid (Krüger et al., 2002). Furthermore, at least five *Rcr3* paralogues are found in *L. pennellii*, spread over ~100 kb (J. Krüger, C. Thomas, C. Golstein and J. Jones, unpublished data). By analogy, a component required for Hcr9 function may belong to a multigene family polymorphic within the *Nicotiana* genus. Thus, the interaction of each Hcr9 auto-activator with specific components encoded by a multigene family could determine their HR-inducing activity in a particular *Nicotiana* species. This differential interaction would appear to depend on subtle differences in the Hcr9 proteins, as the HR-inducing patterns in the five *Nicotiana* species could not be correlated with any obvious amino acid sequences in the auto-activator classes.

The 'polymorphic component' model might be consistent with the guard model (Dangl and Jones, 2001; Van der Biezen and Jones, 1998; Van der Hoorn et al., 2002), which proposes that R proteins monitor (guard) a pathogenicity target (guardee) of their cognate elicitors, rather than interacting directly with these elicitors. Tomato Hcr9s could act as guards of certain *Nicotiana* proteins involved in endogenous processes. Polymorphism in both the Hcr9s and the *Nicotiana* guardees would result in a variety of interactions between

these components, of which some might initiate downstream signalling leading to different levels of HR. This would be observed as Hcr9-mediated auto-necrosis.

Hcr9 auto-activators as a tool to study Cf-4 and Cf-9 function

The auto-activators identified in this study were shown to signal through the Cf-9 pathway, and may therefore be used to study the signal transduction cascade, which is activated by Cf-4 and Cf-9 upon Avr4 and Avr9 recognition, respectively, and leads to HR. The different HR-inducing activities of the auto-activators in the *Nicotiana* testers suggest polymorphism in a component(s) of this signal transduction cascade in these species.

Mutational screens have failed to identify extragenic *Cf-9* null mutants (Hammond Kosack et al., 1994; Wulff et al., 2004). The Avr9 HABS is present in membranes of Solanaceous species (Kooman-Gersmann et al., 1996), and has been postulated to be the guardee of Cf-9 (Joosten and De Wit, 1999). Possibly, the different HR-inducing patterns of the auto-activators in the different *Nicotiana* species may reflect polymorphism in this HABS, which would be consistent with the 'polymorphic component' model.

Similarly, no extragenic null mutants have yet been identified for *Cf-4* (Wulff et al., 2004). For Avr4 a high affinity-binding site of fungal origin was identified (Westerink et al., 2002). Detailed analysis revealed that Avr4 comprises a functional invertebrate chitin-binding domain that protects fungal hyphae against lysis by plant chitinases (Van den Burg et al., 2003). Although Avr4 is specifically recognised in Cf-4 tomato plants, no Avr4-specific binding site could be detected in tomato (Westerink et al., 2002). This suggests that the perception of Avr4 by Cf-4 is also indirect, and that other components should be involved. Therefore, the identification of the proposed polymorphic component(s) responsible for the different auto-activator patterns in *Nicotiana* may be employed as a tool to identify a component required for Cf-4 and/or Cf-9 function.

Previously, a combination of DNA shuffling, classical domain swaps and point mutagenesis was used to determine the specificity requirements of the Cf-4 and Cf-9 resistance proteins, which confer recognition of the fungal elicitors Avr4 and Avr9, respectively. The major specificity determinants were found to be the number of LRRs (Avr4 recognition requires 25 LRRs, whereas Avr9 recognition requires 27 LRRs), a deletion of 10 amino acids in the domain B of Cf-4, and the sequence of putative solvent-exposed amino acids in the central LRRs (Van der Hoorn et al., 2001a; Wulff et al., 2001). Besides *Cf-4*, which originates from *L. hirsutum*, *Hcr9*s that confer recognition of Avr4 have been isolated from four other *Lycopersicon* species and were all found to encode proteins with 25 LRRs, whereas four *Cf-9* alleles encoding 27 LRRs and conferring recognition of Avr9 were isolated from *L. hirsutum* (chapter 4). This confirmed the observed correlation between the number of LRRs and the ability to confer Avr4 or Avr9 recognition. Moreover, the four *Hcr9*s that confer recognition of Avr4 all contained the 10 amino acid deletion in the B-domain (chapter 4). The auto-activator shufflants contain members with either 25 or 27 LRRs, with or without the 10 amino acid deletion in domain B, and diverse amino acid

sequences throughout the LRRs. *Auto 7* and *Auto 10* encode proteins with 25 LRRs (Figure 2), and may therefore be functionally distinct from the other auto-activators. Identification of a component involved in the triggering of HR by these auto-activators may therefore provide clues on how Cf-4 function differs from Cf-9 function.

In conclusion, we describe here a novel class of *R* gene auto-activators, generated by gene shuffling and also occurring naturally. These *Hcr9* auto-activators induce an HR in the absence of fungal elicitors upon transient expression in *Nicotiana* species, and signal through the Cf-9 pathway. The HR-inducing activity of these auto-activators can likely be attributed to different *Nicotiana* factors acting as elicitors, or with different interactions with polymorphic components of the Cf-9 signal transduction cascade. Therefore, these auto-activators may be used to identify components of the Cf-4 and Cf-9 signal transduction cascades that lead to HR.

Materials and Methods

Construction of plasmids containing *Hcr9*s

The plasmids SLJ12574 and SLJ12575 containing *Cf-4* and *Cf-9* fragments extending from a *Cla*I site at the ATG start codon downstream to the internal *Hind*III site have been described (Wulff et al., 2001). The Cf-9[Δ2LRR] construct was also described previously (Wulff et al., 2001). The *4E* clone SLJ13671 was derived from a *4E* containing construct (in which the *Eco*RI and the two internal *Hind*III sites had been removed by mutagenesis without affecting the predicted amino acid sequence (C. Thomas, unpublished data) by mutation of *4E* with primers ClaIHcr9-4E (forward, GCATCGATGGGTTGTGTAAAACTTATATTTTTC, *Cla*I site underlined), to introduce a *Cla*I site at the ATG, and NLC1780R (reverse, TCAAGCTTAAAATCTTCAATTGAGATAGG, *Hind*III site underlined), to re-introduce the conserved 3' *Hcr9 Hind*III site. The resulting PCR product was cloned into pGEM-T (Promega, Southampton, UK) to yield SLJ13671. The *9B* clone SLJ13681 was made by inserting a *Cla*I to *Hind*III fragment from SLJ10601 (Panter et al., 2002) into pBluescript KS^+. SLJ13551 (*9D*) was made by amplifying a 1,792 bp fragment from SLJ10691 (which carries an 8.5 kb genomic *Pst*I fragment containing *9D* without the 3' end cloned in pBluescript II^+ [M. Parniske, unpublished]), with primers ClaI-Hcr90F (forward, GCATCGATGGGTTGTGTAAAACTTGTG, *Cla*I site underlined) and 9D1780R (reverse, CGCGTAAACAAGTTTGTATTCC), digesting the product with *Cla*I and *Hind*III and cloning it in pBluescript KS^+. SLJ13541 (*ΨCf-0*) was constructed by subcloning a 1,161 bp *Sac*I to *Hind*III fragment into pBluescript KS^+ from SLJ13521 (a pBluescript II^+ construct containing a 7.4 kb genomic *Bam*HI *Cf-0* fragment [M. Parniske, unpublished]). SLJ13691 was made by mutating SLJ13501 (which is a pBluescript KS^+ construct containing a 4.6 kb genomic *Eco*RI fragment with the 5' half of *ΨSCB* [M. Parniske, unpublished]), with primers RV-SCB556F (forward, TTTAGATATCCAACTTCACTTCATGAGTTG, to introduce an *Eco*RV site (underlined]) and 9D-1780R. The resulting 894 bp fragment was digested with *Eco*RV and *Hind*III and cloned in pBluescript KS^+. SLJ13702 is a pBluescript KS^+ construct containing a genomic *Pst*I *NLA* fragment (M. Parniske, unpublished), and SLJ13701 is a pBluescript KS^+ construct containing a 2.8 kb genomic *NLC Sal*I fragment (M. Parniske, unpublished). All clones were sequenced to exclude point mutations before being used in shuffling experiments.

The binary expression vectors carrying the 35S driven full length open reading frames of *Cf-4* and *Cf-9* and 3' untranslated regions of *Cf-9*, have been described (Wulff et al., 2001). Full-length *4E*, *9B*, *9D* and *NLC* open reading frames were cloned in the pMOG800 binary expression vector (Honée et al., 1998), under control of the 35S promoter. pMOG800 constructs containing *4E* and *9D* have been described (Westerink et al., 2003;

chapter 3). *9B* and *NLC* were amplified from genomic DNA of the tomato cultivar MoneyMaker-Cf9 with primers DS9 (forward, TTTTTCCATGGGTTGTGTAAAACTTGTG, *Nco*I site underlined) and CS11 (reverse, CCCCCCTGCAGTCACTAATATCTTTTCTTGTGC, *Pst*I site underlined), cloned into pRH80, and subcloned into the pMOG800 vector under control of the 35S promoter as described (Van der Hoorn et al., 2000; chapter 2).

Peru 1 and *peru 2* were amplified from genomic DNA of *L. peruvianum* plants of accessions LA0441 and LA1984 using a degenerate primer mix as described (chapter 4), were subsequently cloned into pRH80 (Van der Hoorn et al., 2000) and subcloned in pMOG800 (Honée et al., 1998) as described (Van der Hoorn et al., 2000; chapters 3 and 4). Several independent clones containing *peru 1* and *peru 2* were sequenced to ensure that no PCR-induced mutations were introduced. The sequences of *peru 1* and *peru 2* have been deposited at GenBank under accession numbers AY634618 and AY634619.

***Hcr9* shuffling**

DNA fragments containing the 5' sequences of *Cf-4*, *Cf-9*, *9B* and *9D* were isolated from plasmids SLJ12574, SLJ12575, SLJ13681 and SLJ13551, respectively, by digestion with *Xho*I and *Sac*I, followed by agarose gel separation and purification. DNA fragments containing *4E*, *ΨCf-0* and *ΨSCB* were isolated from plasmids SLJ13671, SLJ13541 and SLJ13691, respectively, by digestion with *Cla*I and *Hind*III, *Sac*I and *Hind*III, and *Eco*RV and *Hind*III, respectively, followed by gel separation and purification. DNA fragments containing *Cf-9*[Δ2LRR], *ΨNLA*, and *NLC*, were generated by PCR with *Taq* polymerase (Amersham, Chalfont St Giles, UK) on the templates Cf9[Δ2LRR], SLJ13702 and SLJ13701, respectively, with the following primer combinations: 988F (forward, TCCCCAGCTCACGGTTAGGTTTCCC) with 1286R (reverse, GTAACGGTACTTAATGTTTTGG); F77 (forward, GAATCTGTAAACATCTCTTCCACT with NLA1065R (reverse, GCCTTTCAAGTTTCATCCAGCTTCTG); and NLC1004F (forward, GGAGATATCACTTGGAAATAACAATTTGG) with H3NLC1780R (reverse, TCAAGCTTAAAATCTTCAATTGAGATAGG). Excess primers and nucleotides were removed using Qiagen PCR purification columns (Qiagen, Crawley, UK). From the pseudo (Ψ) genes *ΨCf-0*, *ΨSCB* and *ΨNLA*, regions were chosen to exclude the incorporation of their deletions and frameshifts into the shuffled clones. The various *Hcr9* fragments were digested with different combinations of restriction enzymes, as indicated in Figure 1. Two different mixtures of *Hcr9* digests were shuffled separately using *Pfu* DNA polymerase (Promega, Madison, WI) and 15 cycles of primer-free PCR as described (Wulff et al., 2001). Mixture one contained the fragments of *Cf-0* (*Bsp*HI, *Bst*XI, *Eco*RI, *Hae*III, *Ssp*I), *Cf-4* (*Hinf*I, *Tsp*509I), *4E* (*Alu*I, *Nla*III), *9B* (*Hinf*I, *Mnl*I, *Rsa*I), *Cf-9* (*CviJ*I*), *Cf-9*[Δ2LRR] (*Bsm*AI, *Mse*I, *Sty*I), *9D* (*Alu*I, *Tsp*509I), *SCB* (*Ava*I, *Bfa*I, *Dde*I, *Hae*III, *Sna*BI), *NLA* (*Hae*III, *Ssp*I, *Xmn*I) and *NLC* (*Acc*I, *Bfa*I, *Eco*RI, *Pvu*II). Mixture 2 contained the fragments of *Cf-0* (*Bsp*HI, *Bst*XI, *Eco*RI, *Hae*III, *Ssp*I), *Cf-4* (*Alu*I, *Xmn*I), *4E* (*Alu*I, *Nla*III), *9B* (*DpnI*, *Mse*I), *9D* (*Alu*I, *Tsp*509I), *Cf-9* (*Acc*I, *Alu*I, *Bfa*I, *Sau*96I, *Sna*BI), *Cf-9*[Δ2LRR] (*Bsm*AI, *Mse*I, *Sty*I), *SCB* (*Ava*I, *Bfa*I, *Dde*I, *Hae*III, *Sna*BI), *NLA* (*Hae*III, *Ssp*I, *Xmn*I) and *NLC* (*Acc*I, *Bfa*I, *Eco*RI, *Pvu*II). The DNA concentration of each mixture was 10 $ng \cdot \mu l^{-1}$, and the molar ratio of each *Hcr9* before digestion was identical. Upon completion of the first PCR, the two mixtures were combined and a second round of 25 cycles of primer-free PCR was performed as described (Wulff et al., 2001). To recover shuffled *Hcr9* sequences, the PCR products were amplified from aliquots of this mixture as described, using primers SR1 and SF2, which anneal to the pBluescript polylinkers (Figure 1; Wulff et al., 2001). The recovered *Hcr9* gene shuffled products were digested with *Cla*I and *Hind*III and ligated into the binary expression vector pSLJ12904, between the CaMV 35S promoter and the 3' terminal coding sequences and 3' untranslated region of the *Cf-9* gene (Wulff et al., 2001).

***Agrobacterium*-mediated transient gene expression**

pSLJ12904 ligation products containing shuffled *Hcr9*s were electroporated into *A. tumefaciens* strain GV3101 and kanamycin resistant clones were picked into 384-well microtitre plates. *Agrobacterium*-mediated transient

gene expression of all shuffled clones, control constructs, *peru 1* and *peru 2* was performed using *A. tumefaciens* GV3101 in mature leaves of *N. benthamiana* or *N. tabacum* as described (Thomas et al., 2000; Wulff et al., 2001). The same conditions were used for transient expression in *N. knightiana*, *N. rustica* and *N. undulata.*

Plant material

SA depleted *N. benthamiana* plants were made by transformation of wild type *N. benthamiana* plants with *A. tumefaciens* strain LBA4404 harbouring the binary T-DNA construct SLJ7321, which carries a 35S:omega leader:*nahG* gene with a *nos* terminator sequence (Brading et al., 2000), as described by Horsch et al. (1985). Primary transformants carrying a single T-DNA integration were selected by plating of T_2 progeny seeds on Murashige and Skoog (1962) medium containing 100 $\mu g \cdot ml^{-1}$ kanamycin, and the ratio of kanamycin-resistant to -sensitive seedlings was determined. Single copy transformants were analysed for expression of the *nahG* gene by northern blot analysis, and one line, L-20266-1-J5, with the highest *nahG* mRNA level was selected. Homozygous L-20266-1-J5 plants did not accumulate detectable SA levels (determined by HPLC analysis) and did not show induction of the PR genes *PR1a* and *PR2a* (determined by RT-PCR analysis) 24 h after infiltration of 1 mM SA into leaves.

L. peruvianum LA0441 and LA1984 seeds were obtained from Roger Chetelat of the Tomato Genetic Resource Center, Davis, CA. Seeds of *N. knightiana* 889G, *N. rustica* 899E and *N. undulata* AW125 were obtained from Mike Ambrose, John Innes Centre, Norwich, UK.

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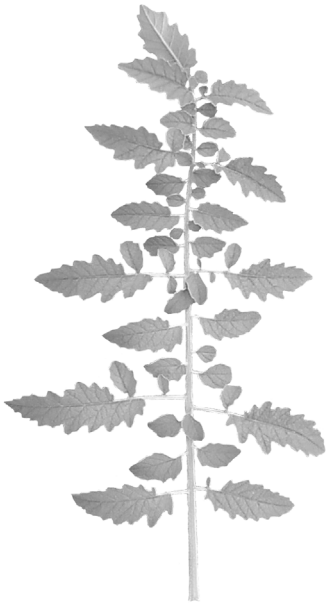
Chapter 6

General discussion:

Receptor-like protein encoding resistance genes in plants



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To be submitted for publication.

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General discussion:

Receptor-like protein encoding resistance genes in plants

Plants are attacked by many pathogenic organisms including viruses, bacteria, fungi, and nematodes. Plants resist these pathogen attacks both by preformed defences such as antimicrobial secondary compounds and by induced defence responses (Heath, 2000). Inducible defences can be activated upon recognition of general elicitors, such as bacterial flagellin (Gomez-Gomez and Boller, 2002; Zipfel et al., 2004). In addition, plants have evolved sophisticated recognition systems to detect specific pathogen elicitor proteins produced during infection. Recognition of these specific elicitors, referred to as avirulence (Avr) proteins, is mediated by plant disease resistance (R) proteins in a highly specific manner, which has been genetically defined as the gene-for-gene interaction (Flor, 1946, 1971). The identification of many *R* genes, and in many cases also the corresponding *Avr* genes, has contributed greatly to our understanding of the molecular basis of gene-for-gene disease resistance (Martin et al., 2003; Van 't Slot and Knogge, 2002).

The majority of *R* genes encode proteins that mediate Avr protein recognition fall into five classes, based on the presence of a limited number of structural motifs (Figure 1). Class 1 consists of only one member, Pto from tomato, which has a serine/threonine kinase catalytic domain and a myristylation motif (Martin et al., 1993). The second class comprises a large number of proteins from diverse plant species and carry a leucine-rich repeat (LRR) domain, a putative nucleotide binding site (NBS) domain and an N-terminal putative leucine-zipper (LZ) or coiled-coil (CC) domain (reviewed by Martin et al., 2003). Class 3 is similar to class 2, but instead of the CC domain, class 3 proteins have a domain that bears similarity to the N-terminus of the Toll and Interleukin 1 receptor, referred to as the TIR domain. The R proteins belonging to the first three classes are all predicted to be intracellular (Martin et al., 2003). Class 4 contains the receptor-like proteins (RLPs), which have an extracellular LRR (eLRR) domain, a single transmembrane (TM) domain, and a small cytoplasmic tail. This group is mainly formed by the tomato Cf proteins (reviewed by Joosten and De Wit, 1999). Finally, class 5 consists of the Xa21 protein from rice and the Arabidopsis FLS2 protein, which, in addition to an eLRR domain and a TM domain, have a cytoplasmic serine/threonine kinase domain (Gomez-Gomez and Boller, 2000; Song et al., 1995). A few R proteins do not fall into these five classes (Martin et al., 2003). They may act in specific recognition or play a role in detoxification of pathogen-derived toxins, but have different combinations of structural motifs or completely novel structures (Martin et al., 2003; Nimchuk et al., 2003).

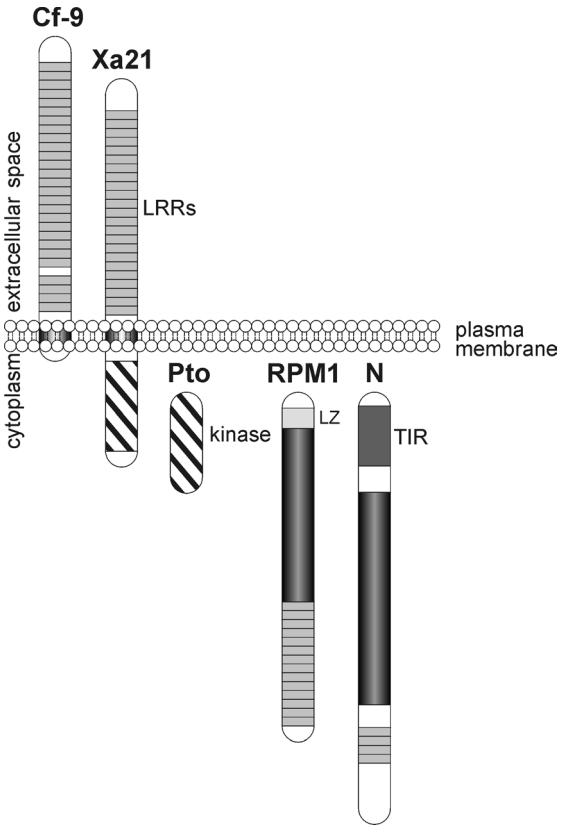

Figure 1. Schematic representation of the five major structural classes of plant R proteins.
One R protein of each class is represented. Cf-9, *Cladosporium fulvum* resistance protein of *Lycopersicon pimpinellifolium* (currant tomato) (Jones et al., 1994); Xa21, *Xanthomonas oryzae* pv. *oryzae* resistance protein of *Oryza sativa* (rice) (Song et al., 1995); Pto, *Pseudomonas syringae* pv. *tomato* resistance protein of *L. pimpinellifolium* (Martin et al., 1993); RPM1, resistance protein against *P. syringae* pv. *maculicola* of *Arabidopsis thaliana* (Grant et al., 1995); N, resistance protein against Tobacco Mosaic Virus of *Nicotiana tabacum* (tobacco) (Whitham et al., 1994). LRRs, leucine-rich repeats; TIR, Toll/Interleukin-like receptor domain; LZ, leucine-zipper; NBS, nucleotide-binding site. See text for further details.

In this review, we focus on resistance genes that encode class 4 proteins, with emphasis on the well-studied *Cf* genes from tomato that mediate resistance against the biotrophic fungus *Cladosporium fulvum* (Joosten and De Wit, 1999). Tomato *Cf* genes have long been the only known RLP resistance genes in plants. However, recent studies on other plant-pathogen interactions have also identified *Cf*-like resistance genes in other plant species. We present the state of the art of research on the *Cf* genes involved in resistance in tomato to *C. fulvum*. We discuss the organisation and evolution of *Cf* gene clusters, Cf protein structure and specificity, and signal transduction pathways that are initiated upon elicitor perception. We subsequently describe other pathosystems in which RLP genes mediate disease resistance, and the role of RLP genes in other biological processes.

Characteristics of RLP genes involved in plant disease resistance

Tomato *Cf* genes against *Cladosporium fulvum*

The interaction between tomato and the biotrophic leaf fungus *C. fulvum* provides an ideal model system to study RLP resistance genes. During infection of tomato, *C. fulvum* does not penetrate plant cells, but grows in the extracellular space of tomato leaves. As a result, all communication between the fungus and its host plant is restricted to the extracellular space. De Wit and Spikman (1982) showed that specific elicitor proteins are present in the

apoplastic fluids (AFs) of infected susceptible plants. From these AFs several elicitor proteins have been isolated from which the corresponding genes have been cloned and characterised (Luderer et al., 2002; Westerink et al., 2003; reviewed by Joosten and De Wit, 1999). All *C. fulvum* elicitors are small, cysteine-rich proteins, which can be grouped into two classes. The first class comprises the race-specific Avr proteins. Some *C. fulvum* strains do not produce these Avrs, and therefore avoid detection in tomato plants that carry the matching *Cf* genes. These virulent *C. fulvum* strains appeared after large-scale deployment of *Cf* genes. Detailed analysis of the *Avr2*, *Avr4*, *Avr4E* and *Avr9* genes has revealed that the fungus employs several distinct mechanisms to avoid recognition mediated by *Cf* genes. These include (i) point mutations leading to the production of unstable or non-functional elicitor proteins, (ii) frame-shift or non-sense mutations in the *Avr* genes, (iii) complete deletion of the *Avr* gene, and (iv) insertion of a transposon in an *Avr* open reading frame (Joosten et al., 1994; Luderer et al., 2002; Van Kan et al., 1991; Westerink et al., 2003). For most Avr proteins no function is known yet, but for the Avr4 protein it was recently shown that it binds to chitin fragments and might play a role in protecting fungal hyphae against plant chitinases (Van der Burg et al., 2003).

The second class comprises the Extracellular proteins (Ecps), which are secreted by all known *C. fulvum* strains during infection, and race-specificity has not yet been observed (Laugé et al., 2000; reviewed by Joosten and De Wit, 1999). Matching *Cf* genes, designated *Cf-Ecp* genes, have been identified in wild tomato genotypes (Laugé et al., 2000). Sequence variation within the *Ecp* genes is largely absent (De Kock et al., unpublished data). This probably reflects the fact that the *Cf-Ecp* genes have not been deployed in large-scale breeding programs, or suggests that the *Ecp* genes play an important role in pathogenicity, as was observed for *Ecp1* and *Ecp2* (Laugé et al., 1997). Essential virulence factors contribute to the fitness of the pathogen. Therefore, loss of these essential virulence factors will result in a fitness penalty for the pathogen, and resistance based on recognition of these factors is expected to be more durable.

The tomato *Cf* genes group into two large gene families. The *Cf-4*, *Cf-4E*, *Hcr9-9B, Cf-9,* and *9DC* genes are highly homologous and belong to the *Hcr9* (Homologues of *C. fulvum* resistance gene *Cf-9*) gene family (Jones et al., 1994; Laugé et al., 1998a; Parniske et al., 1997; Takken et al., 1998; Thomas et al., 1997; chapter 2). Similarly, the *Cf-2* and *Cf-5* genes belong to the *Hcr*2s (Homologues of the *C. fulvum* resistance gene *Cf-2*) (Dixon et al., 1996, 1998). Both classes are defined by a high overall DNA and protein identity, and both contain functional *Cf* genes and members with unknown functions. *Cf*-mediated recognition of an elicitor protein will invoke defence responses including a hypersensitive response (HR), which eventually leads to complete inhibition of fungal growth.

Genetics and evolution of tomato *Cf* genes

Of all *Cf*-like resistance genes that have been cloned thus far, the largest group comprises the *C. fulvum* resistance genes from tomato. Whereas *Cf-2* and *Cf-5* map on chromosome 6 of

tomato (Dickinson et al., 1993), *Cf-4*, *Cf-4E*, *Hcr9-9B*, *Cf-9, 9DC*, *Cf-Ecp2*, *Cf-Ecp3*, and *Cf-Ecp5* all map on the short arm of chromosome 1 (Figure 2) (Balint-Kurti et al., 1994; Haanstra et al., 1999, 2000a; Jones et al., 1993; Parniske et al., 1997; Takken et al., 1998; Yuan et al., 2002; chapter 3). Although the *Hcr2*s and *Hcr9*s fall into two distinct groups, they are most likely derived from a common ancestral gene and were separated by translocation to different chromosomes. Likely, subsequent independent evolution has resulted in the two distinct groups of *Cf* homologues known today.

The *Hcr2* gene family thus far comprises two near-identical *Cf-2* genes, the *Cf-5* gene and six homologues that are not involved in *C. fulvum* resistance (Figure 2A) (Dixon et al., 1996, 1998). The *Cf-2/5* locus of the susceptible tomato cultivar MoneyMaker-Cf0 carries two *Hcr2*s without a known function in *C. fulvum* resistance (Dixon et al., 1998). The *Cf-2* cluster, which originates from *Lycopersicon pimpinellifolium*, comprises three *Hcr2*s, including the two *Cf-2* genes. *Cf-2-1* and *Cf-2-2* encode proteins that differ only by three amino acids and both confer resistance to *C. fulvum* isolates that produce the Avr2 elicitor (Dixon et al., 1996; Luderer et al., 2002). The two *Cf-2* genes are likely the result of a recent duplication event. The *Cf-5* cluster of *L. esculentum* var. *cerasiforme* contains four *Hcr2*s, of which *Hcr2-5C* is the functional *Cf-5* gene (Dixon et al., 1998). The *Hcr2-5D* gene encodes a protein with two additional LRRs compared to *Cf-5*, and further differs only by two amino acids flanking these two LRRs. *Hcr2-5D* is expressed, but despite its high homology with *Cf-5*, does not confer resistance to *C. fulvum* strains that produce Avr5 (Dixon et al., 1998). Not all three *Hcr2* loci have been sequenced completely, but *Hcr2-0A*, *Hcr2-2A*, and possibly *Hcr2-5A*, appear to be orthologous, whereas the other *Hcr2*s fall into another group. This suggests that duplication and divergence of the *Hcr2*s has occurred before speciation (Dixon et al., 1998).

All *Hcr9*s are likely derived from a single *Hcr9* progenitor gene, as was shown in a study by Parniske and co-workers (1997), who isolated the *Cf-0*, *Cf-4* and *Cf-9* gene clusters. All three clusters map to the *Cf-4/9 Milky Way* (*MW*) locus. Whereas the *L. esculentum Cf-0* cluster consists of only a single homologue without a known function in *C. fulvum* disease resistance, the *Cf-4* and *Cf-9* clusters both comprise five homologues (Figure 2B). The *Cf-4* cluster from *L. hirsutum* contains five *Hcr9*s of which two function as *C. fulvum* resistance genes (Figure 2B). The *Hcr9-4D* homologue is the *Cf-4* gene that mediates recognition of Avr4 (Thomas et al., 1997), and *Hcr9-4E* is the *Cf-4E* gene that mediates recognition of the Avr4E elicitor (Takken et al., 1998; Westerink et al., 2003). In the *Cf-9* cluster from *L. pimpinellifolium*, the *Hcr9-9C* homologue is the *Cf-9* gene that mediates recognition of Avr9 (Jones et al., 1994), and the *Hcr9-9B* gene provides partial resistance against *C. fulvum*, possibly by mediating recognition of the yet unidentified Avr9B elicitor (Laugé et al., 1998a; Parniske et al., 1997). Analysis of these three clusters showed that positive selection for diversification has acted on residues at putative solvent-exposed positions in the first 17 LRRs of the *Hcr9*s, consistent with the idea that specificity for elicitor recognition resides in the N-terminal half of Cf proteins (Dixon et al., 1996; Parniske et al., 1997).

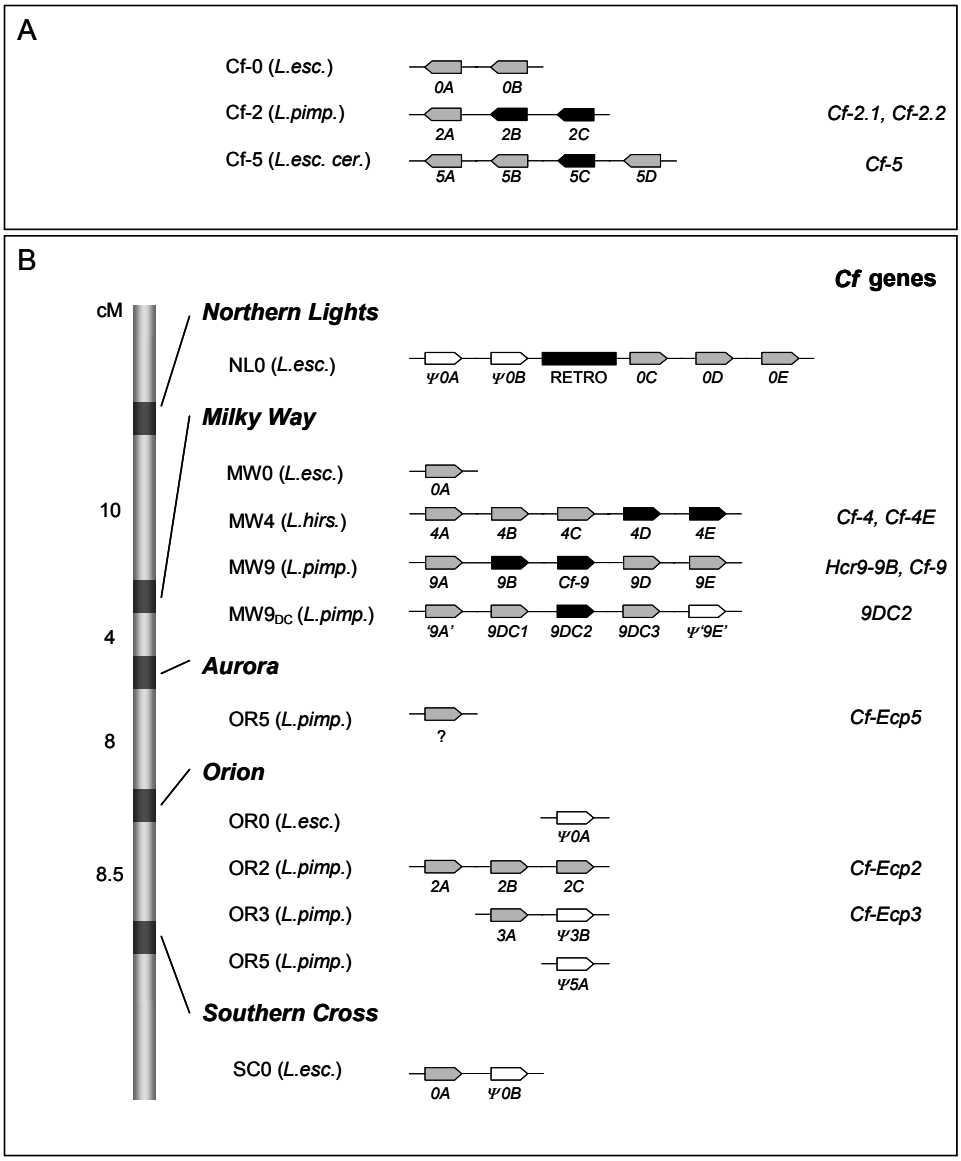

Figure 2. Overview of the physical structure of all isolated *Hcr2* and *Hcr9* gene clusters, and map positions of all *Hcr9* clusters.

A. Physical structure of *Hcr2* gene clusters on chromosome 6 of different *Lycopersicon* haplotypes. Cf-0: *L. esculentum*; Cf-2: *L. pimpinellifolium*; Cf-5: *L. esculentum* var. *cerasiforme*.

B. Map position, genetic distance (cM) and physical structure of the *Northern Lights*, *Milky Way*, *Aurora*, *Orion* and *Southern Cross* loci harbouring clusters of *Hcr9*s on the short arm of chromosome 1. On the left, a genetic map of the various clusters showing the position of the five *Hcr9* loci relative to each other. In the middle, the physical organisation of each *Hcr9* cluster is shown. On the right, the *Cf* resistance genes present in the cluster are indicated. Arrowed boxes indicate the relative position and orientation of *Hcr9*s; white arrowed box, *Hcr9* pseudogene; grey arrowed box, *Hcr9* with unknown function; black arrowed box, functional *Hcr9* resistance gene. *Hcr9* clusters are derived from different haplotypes: NL0, *L. esculentum* Cf0; MW0, *L. esculentum* Cf0; MW4, *L. hirsutum* Cf4; MW9, *L. pimpinellifolium* Cf9; $MW9_{DC}$, *L. pimpinellifolium* 9DC; OR0, *L. esculentum* Cf0; OR2, *L. pimpinellifolium* Cf-Ecp2; OR3, *L. pimpinellifolium* Cf-Ecp3; OR5, *L. pimpinellifolium* Cf-Ecp5; SC0, *L. esculentum* Cf0. The organisation of the *Aurora* cluster is unknown. RETRO denotes a retrotransposon insertion in the *NL* haplotype.

Furthermore, a patchwork of sequence similarities was revealed in the *Hcr9* family members, strongly suggesting extensive sequence exchange between the *Hcr9*s. Therefore, sequence exchange between *Hcr9*s, rather than accumulation of point mutations, was proposed as the main mechanism by which novel *Hcr9* genes are generated (Parniske et al., 1997). Sequence exchange between *Hcr9*s may ultimately lead to homogenisation of the *Hcr9*s within a cluster. Polymorphism in the intergenic regions between *Hcr9*s is thought to play a pivotal role in preventing homogenisation. In a homozygous background, they suppress unequal recombination between sister chromatids, whereas between suitable heteroallelic haplotypes alignment of homologous sequences and recombination is permitted (Parniske et al., 1997). Such unequal recombinations alter the *Hcr9* number and composition of the clusters, and therefore lead to an increase of haplotype variation in the population.

In another study, the isolation of two *Hcr9* clusters from *L. esculentum* that reside proximal and distal to the *MW* locus was described (Parniske and Jones, 1999). The *Northern Lights* (*NL*) cluster consists of five *Hcr9*s (Figure 2B). Whereas the *ΨNL0A* (Ψ-prefix indicates a pseudogene), *ΨNL0B*, *NL0D* and *NL0E* genes form a diverged subclass of *Hcr9*s, the *NL0C* gene appears to belong to the *MW* subclass. The *NL0C* gene is likely the result of ectopic recombination between the *MW* and *NL* clusters. The *Southern Cross* (*SC*) cluster contains only two *Hcr9*s (*SC0A* and *ΨSC0B*), which belong to the *MW* subclass (Figure 2B). This indicates that the *NL* cluster, with the exception of the *NL0C* homologue, was physically separated by translocation from the *MW* and *SC* cluster before the *MW* and *SC* clusters were separated (Parniske and Jones, 1999).

Cf-9 originates from *L. pimpinellifolium*, and in a study on *Cf-9* variation in the *L. pimpinellifolium* population the *9DC* gene was discovered (chapter 2). This gene has the same specificity and activity in conferring Avr9 responsiveness as *Cf-9*, and is related to *Cf-9* by an intragenic recombination. This provides a clear example of sequence exchange between *Hcr9*s leading to novel *Cf* genes. In six additional *Cf-9* alleles no polymorphism was found, but the *9DC* gene exhibited limited polymorphism. Furthermore, the *9DC* gene occurred at a higher frequency in the *L. pimpinellifolium* population than *Cf-9*. This suggested that *9DC* might be ancestral to *Cf-9* (chapter 2). Recently, the complete *9DC* cluster was isolated from *L. pimpinellifolium* LA1301, and it was mapped at the *MW* locus (Figure 2B) (chapter 3). Therefore, two *Hcr9* clusters originating from the same species and locus and with the same specificity could be compared. The *9DC* cluster has a similar organisation as the *Cf-9* cluster, and contains *Hcr9-9A* and *Hcr9-9E* orthologues, a *Cf-9* fragment, part of an *Hcr9-9B* orthologue fused to another *Cf-9* fragment, and three *9DC* genes (Figure 3A). Detailed analysis of both clusters strongly suggested that *Cf-9* and the adjoining *Hcr9-9D* homologue are ancestral to *9DC*, in contrast to previous suggestions (chapter 2). The recombination event that created the first *9DC* gene would have generated two identical intergenic regions, which likely increased the probability of mispairing of individual homologues and unequal recombination. Two additional *9DC* genes were likely generated by several intra- and

intergenic recombination events, leading to identical *9DC1* and *9DC2* open reading frames. The *9DC3* gene is likely the result of recombination between a third identical *9DC* gene and an *Hcr9-9D* orthologue (chapter 3). All three *9DC* genes confer responsiveness to Avr9 in tobacco under control of the 35S promoter, but only *9DC2* is active under control of its native promoter. This suggested that *9DC2* is the main determinant of Avr9 recognition in *L. pimpinellifolium* LA1301 (chapter 3).

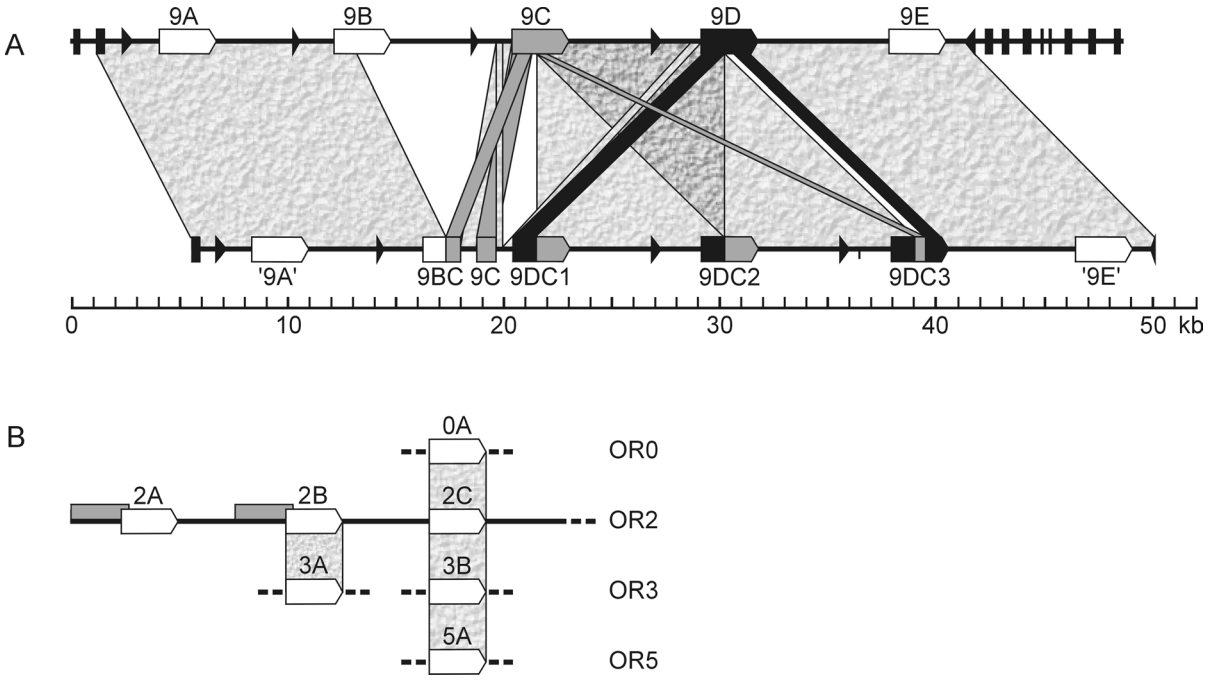

Figure 3. Schematic representation of the relationships between the *Cf-9* and *9DC* clusters, and between the *Orion Hcr9*s.

A. Relationships between the *Cf-9* (top) and *9DC* (bottom) clusters. Arrowed boxes represent complete *Hcr9*s; *Cf-9* (*9C*)-like sequences are depicted in grey; *Hcr9-9D* (*9D*)-like sequences in black; other *Hcr9* sequences are depicted in white. All *Hcr9*s and *Hcr9* fragments in the *Cf-9* and *9DC* clusters are in the 5'-3' orientation. Black arrows and bars represent *LipoxygenaseC* exons and the arrows indicate the polarity of transcription of the 3'-exon. Grey marbled boxes connecting the *Cf-9* and *9DC* clusters indicate orthologous regions. Orthologous regions of the *Cf-9* and *9D* genes are connected by grey and black boxes, respectively. Note that in the central part of the *9DC* cluster an 8.7 kb repeat is present that is near-identical to a region in the *Cf-9* cluster (over 99.9% identical). The connecting boxes between this region of the *Cf-9* cluster and of the two regions in the *9DC* cluster overlap in the dark grey marbled triangle. The three *9DC* genes are likely the result of several intra- and intergenic recombinations between *Cf-9* and *Hcr9-9D*. This figure has been adapted from chapter 3.

B. Relationships between the *Orion* (*OR*) *Hcr9*s The *OR* cluster of the Cf-Ecp2 genotype (OR2) has been sequenced completely. The composition of the *OR* clusters of the other three genotypes (Cf-0, Cf-Ecp3, Cf-Ecp5) was determined by *Hcr9*-fingerprinting and locus-assignment, and only the *Hcr9* open reading frames (ORFs) were sequenced. The grey marbled boxes indicate orthologous *OR Hcr9*s (93% DNA sequence identity between *OR2B* and *OR3A*; 99.5-99.9% DNA sequence identity between *OR2C* and its orthologues). Dark grey boxes indicate a perfect 2.6 kb duplication that comprises 2.2 kb sequence upstream of the *OR2A* and *OR2B* ORFs and the first 0.4 kb of the ORFs of these genes. Data were obtained from De Kock et al. (unpublished data).

In a genus-wide screen for functional homologues of *Cf-4* and *Cf-9*, comprising all wild tomato species, many Avr4- and Avr9-responsive tomato plants were identified in different species (chapter 4). Avr4 recognition was observed in all eight wild species, except *L. pimpinellifolium*, whereas Avr9 recognition was observed in a smaller number of species (Figure 4). From five different species *Hcr9*s that confer Avr4 responsiveness (designated *Hcr9-Avr4*s) were isolated, including *Cf-4* from *L. hirsutum*. The *Hcr9-Avr4*s are over 96% identical, compared to a minimum of 73% for all *Hcr9*s. All features previously identified to be essential for Cf-4 function (Van der Hoorn et al., 2001a; Wulff et al., 2001) are conserved in the five Hcr9-Avr4 proteins. From *L. hirsutum*, four *Hcr9*s that confer Avr9 responsiveness (designated *Hcr9-Avr9*s) were isolated, that all represent *Cf-9* alleles. *Cf-9* and the Hcr9-*Avr9s* from *L. hirsutum* are over 98% identical at the DNA level, and *Hcr9-Avr9-hir1* differs only by a single silent mutation from the *L. pimpinellifolium Cf-9* gene. *Hcr9-Avr9-hir4* encodes the most diverged protein, with 22 amino acid substitutions relative to Cf-9. The high sequence conservation of the *Hcr9-Avr4*s and *Hcr9-Avr9*s in diverged *Lycopersicon* species led to the conclusion that these genes predate *Lycopersicon* speciation, and apparently provide a selective advantage in natural *Lycopersicon* populations. This notion was further supported by the identification of two Avr9-responsive *Solanum* species, which are closely related to the *Lycopersicon* genus (Laugé et al., unpublished data; chapter 4). This further suggests that *C. fulvum* is an ancient pathogen of the *Lycopersicon* genus, and has exerted a selection pressure for maintaining functional *Cf* genes.

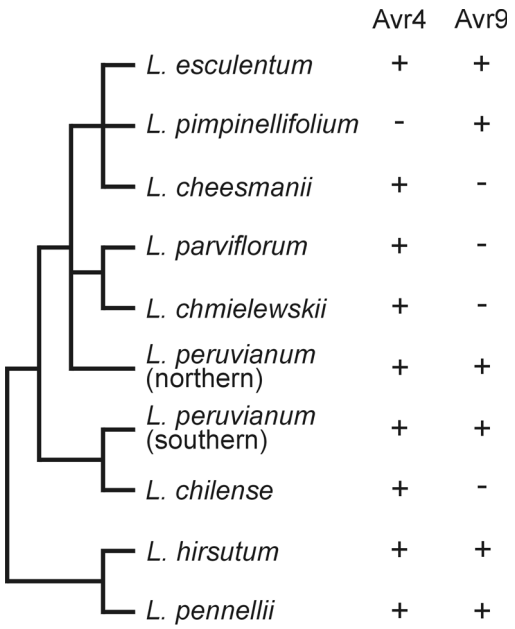

Figure 4. Tentative representation of phylogenetic relationships between the *Lycopersicon* species and Avr4 and Avr9 responsiveness within these species. Avr4 and Avr9 responsiveness was introduced in cultivated tomato (*L. esculentum*) from wild tomato species. This figure is adapted from chapter 4.

Three *Cf-Ecp* genes were mapped and found to co-segregate with *Hcr9*s. The *Cf-Ecp2* and *Cf-Ecp3* genes are located at the *Orion* (*OR*) locus (Haanstra et al., 1999; Yuan et al., 2002) whereas *Cf-Ecp5* maps at the *Aurora* (*AU*) locus (Haanstra et al., 2000a, 2000b). The Cf-Ecp2 *OR* cluster contains three tandemly repeated *Hcr9*s (Figure 2B), including a 2.6 kb tandem repeat, that covers the promoter region and the first part of the coding region of *OR2A* and *OR2B* (Figure 3B) (De Kock et al., unpublished data). This duplication is probably the result of a recent intergenic unequal recombination. Orthologous *Hcr9*s were identified in different *Lycopersicon* species and haplotypes (Figure 2B) (De Kock et al., unpublished data). The Cf-Ecp3 haplotype contains two *Hcr9*s at the *OR* locus, and the Cf-0 and Cf-Ecp5 *OR* loci both contain only one *Hcr9* at the *OR* locus. *OR0A* is present in the Cf-0 *L. esculentum* haplotype, whereas the other genes originate from different *L. pimpinellifolium* accessions. Complementation analysis with candidate *Cf-Ecp2* and *Cf-Ecp3* genes has yet to reveal which genes mediate the perception of the *C. fulvum* elicitors Ecp2 and Ecp3, respectively.

The orthologous genes of *OR2C* (Ψ*OR0A*, Ψ*OR3B* and Ψ*OR5A*) are almost identical, whereas the other *OR Hcr9*s are very homologous (Figure 3B). Based on the shared polymorphic sites, the *OR Hcr9*s are most related to *ΨNL0A*, *ΨNL0B, NL0D* and *NL0E*. In contrast, the intergenic regions appear to be unique for the *OR* locus. The *MW* and *SC* clusters harbour several *LipoxygenaseC* (*LoxC*) exons that are thought to have co-duplicated with *Hcr9*s (Parniske et al., 1997). These *LoxC* sequences are absent in both the *NL* and the *OR* cluster. Moreover, the *OR Hcr9*s and the *NL Hcr9*s are more homologous to each other than to the *Hcr9*s of the *MW* and *SC* clusters. Therefore, this does not support the suggestion by Parniske and Jones (1999) that the divergence of the *NL Hcr9*s was probably a consequence of their genetic isolation.

In conclusion, *Cf* genes are generally located in clusters of tandemly repeated homologues. A few homologues encode functional resistance genes active against *C. fulvum,* but the other homologues may represent a reservoir of variation that may be employed in the generation of novel *Cf* genes. Duplications, translocations, intra- and intergenic recombinations, gene-conversions and point mutations have all been reported. The major mechanism of generation of novel variation appears to be sequence exchange between homologues. Although the tandemly repeated structure allows large diversification in *Cf* homologues, functional *Cf* genes appear to be highly conserved in many different tomato species. This implies that *C. fulvum* is an ancient pathogen of tomato that has applied selection pressure for maintenance of these functional *Cf* genes in wild tomato populations.

Tomato Cf protein characteristics

Cf proteins contain a putative signal peptide for extracellular targeting (A-domain) which is followed by a cysteine rich B-domain with no known function, an eLRR domain with an internal loop-out structure (C-domain), a D-domain without conspicuous features, an acidic E-domain, a putative transmembrane domain (F-domain) and a short, basic G domain (Jones

et al., 1994; Jones and Jones, 1997) (see Figure 3B in chapter 1). The structure of the domains predicts that Cf proteins are anchored in the plasma membrane, that domains B-E are extracellular and that the G-domain is cytoplasmic.

In contrast to the Hcr9s, of which most carry 27 LRRs, the number of LRRs in the Hcr2s is more variant. For example, the Cf-2 proteins contain 38 LRRs, and the Hcr2-2A and Hcr2-5B proteins contain 25 LRRs. Most of the LRRs of Hcr2 proteins can be classified into two subgroups (A and B), which alternate to give a second level of repeated structure. Due to this repetitive nature, these *Hcr2* repeats are prone to intragenic recombinations, which may alter the number of LRRs and contribute to the variation in the *Hcr2*s, and therefore may generate novel *Cf* genes.

Typical for Hcr2 and Hcr9 proteins, the majority of the amino acid variation between members of this family is found in the B-domain and in the putative solvent exposed residues of the first 17 LRRs. Several studies have identified regions of specificity in the Cf-4 and Cf-9 proteins by exchanging sequences within the first 17 LRRs between these proteins (Van der Hoorn et al., 2001a; Wulff et al., 2001). The number of LRRs was found to be essential for both Cf-4 (25 LRRs) and Cf-9 (27 LRRs) function. Whereas sequences specifically required for Cf-9 function appear to be distributed over a large number of LRRs, specificity in Cf-4 could be pinpointed to a specific 10 amino acid deletion in the B-domain and three solvent-exposed residues in LRRs 11, 12 and 14 (Van der Hoorn et al., 2001a; Wulff et al., 2001). A similar study has shown that the number of LRRs also determines the specificity of the Cf-2 and Cf-5 proteins (Seear and Dixon, 2003).

Overall comparison of the Hcr2 and Hcr9 proteins reveals remarkable homology in their C-termini, particularly in the last 9.5 C-terminal LRRs. This suggests that the conserved C-terminal eLRRs of Hcr2s and Hcr9s interact with a shared component of the signal transduction cascade that initiates the defence responses, whereas the N-terminal domains may play a role in specific recognition (Dixon et al., 1996, 1998). In contrast, amino-acid variation in the *OR* Hcr9s is spread throughout the protein, as here variation is also found in the C-terminal LRRs 18-24, the loop-out and the acidic E-domain. Based on these differences, the *OR* Hcr9s possibly comprise a different class of Hcr9 proteins.

At the C-terminus of Hcr9s a dilysine motif for targeting to the endoplasmic reticulum (ER) is present. The subcellular location of Cf-9 has been a matter of debate for some time. When overexpressed in tobacco and Arabidopsis the dilysine motif in Cf-9 was apparently active, suggesting that Cf-9 might reside in the ER (Benghezal et al., 2000). However, another study showed that the functional Cf-9 protein was localised in the plasma membrane (Piedras et al., 2000). Mutation of the dilysine motif showed that the ER retrieval-retention is not required for Cf-9 function and may be masked by interacting proteins (Van der Hoorn et al., 2001b). Therefore, active Cf-9 is most likely located at the plasma membrane. However, when Cf-9 is overexpressed, it is possible that not all Cf-9 ER-retrieval motifs are masked, and that the bulk of the Cf-9 protein is targeted to the ER. In addition, the presumed TM of Hcr2 and Hcr9 proteins (Jones et al., 1994) appears to contain the YXXΦ endocytosis signal

(Φ represents an amino acid with a hydrophobic side chain, X represents any amino acid), which can stimulate receptor-mediated endocytosis and degradation of mammalian cell-surface receptors (Bonifacino and Traub, 2003; Letourneur and Klausner, 1992). This suggests that the endocytosis motif resides in the cytoplasmic tail rather than in the TM, and that the TM is shorter than initially presumed. The endocytosis signal could provide a mechanism by which cells selectively capture ligands, and may regulate responsiveness to these ligands by regulating the concentration of Hcr proteins on the plasma membrane via controlled breakdown.

Elicitor perception mediated by tomato Cf proteins

Based on the gene-for-gene model, R proteins and Avr factors are predicted to interact, either directly or indirectly (Gabriel and Rolfe, 1990; Van der Biezen and Jones, 1998). A physical interaction between R proteins and Avr proteins has been demonstrated in a few cases (Jia et al., 2000; Leister and Katagiri; 2000; Ron and Avni, 2004; Scofield et al., 1996; Tang et al., 1996), but seems to be the exception (Van der Hoorn et al., 2002). It is not yet known how Cf proteins activate downstream signalling components. However, it has become apparent that the interaction between Cf and Avr proteins is most likely indirect. Several experiments failed to show a direct interaction between Cf-9 and Avr9 (Luderer et al., 2001), and it is likely that Cf-9-mediated 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). Mutagenesis of a Cf-2 tomato line has identified the secreted papain-like cysteine endoprotease Rcr3, which is specifically required for Cf-2/Avr2-mediated HR function (Dixon et al., 2000; Krüger et al., 2002). The *L. esculentum* allele of *Rcr3* (*Rcr3*^{esc}) actively induces a *Cf-2*-dependent auto-necrosis, whereas the *L. pimpinellifolium* allele (*Rcr3*^{pim}) that was co-introgressed with *Cf-2* into cultivated tomato, can suppress this auto-necrosis. As Rcr3 is a secreted cysteine protease with a specific role in *Cf-2*-mediated resistance, it likely functions upstream of Cf-2. Rcr3 might process Avr2 to produce a mature ligand, or Cf-2 or another plant protein. Alternatively, Rcr3 might be a plant defence component that is specifically inhibited by Avr2, or Rcr3 and Avr2 together constitute a complex ligand that is recognised by Cf-2 (Krüger et al., 2002). For the Avr4 elicitor protein from *C. fulvum* a high affinity-binding site of fungal origin was identified, whereas in tomato an Avr4-specific binding site could not be detected (Westerink et al., 2002). Detailed analysis revealed that Avr4 comprises a functional invertebrate chitin-binding domain that protects fungi against lysis by plant chitinases (Van den Burg et al., 2003). Mutant Avr4 isoforms are no longer recognised in Cf-4 tomato plants, but have retained their chitin-binding activity (Van den Burg et al., 2003).

Cf-4- and Cf-9-mediated HR was shown to be temperature sensitive, possibly due to decreased levels of Avr-binding proteins (De Jong et al., 2002). This suggests that the formation of Cf-complexes is hampered at elevated temperatures. Candidate proteins that might be present in such Cf-complexes are receptor-like kinases (RLKs) (Joosten and De

Wit, 1999), similar to CLAVATA-1 (CLV1), which forms a complex with the RLP CLV2 and functions in determining cell fate in meristems (Torii, 2000). Attempts to purify Cf-4 and Cf-9 complexes using gel filtration (Rivas et al., 2002a, 2000b) were unsuccessful, as Cf-4 and Cf-9 appeared to migrate as a large complex during gel filtration, which was shown to be an intrinsic property of the Cf proteins themselves (Van der Hoorn et al., 2003).

Taken together, these data suggest that the interaction between Cf and Avr proteins is, at least mostly, indirect, and that Cf proteins may function as guards of the virulence targets of the Avr proteins (Van der Biezen and Jones, 1998; Van der Hoorn et al., 2002). *Cf* genes have also been shown to be functional in other Solanaceous species (Hammond-Kosack et al.; 1998; Thomas et al., 2000; Van der Hoorn et al., 2000). Moreover, the Avr9 HABS was also found in several Solanaceous plants (Kooman-Gersmann et al., 1996), and Avr9-responsive plants were identified in two different *Solanum* species (Laugé et al., unpublished data; chapter 4) suggesting that the full signal transduction cascade required for Cf protein mediated signalling is conserved throughout the Solanaceae.

Signal transduction mediated by tomato Cf proteins upon elicitor recognition

Upon Avr protein recognition, *Cf* gene products activate a signal transduction cascade that leads to activation of plant defence responses, including an HR (Hammond-Kosack and Jones, 1996). Many experiments that address early signalling events in *Cf*-mediated HR were performed using Cf-4 or Cf-9 expressing tobacco cell suspensions, as *Cf-9* tomato cell suspensions failed to initiate defence responses upon Avr9 treatment (Honée et al., 1998). Avr9 treatment of *Cf-9* tobacco cells results in the formation of active oxygen species (oxidative burst), ion fluxes and medium alkalisation (Blatt et al., 1999; De Jong et al., 2000; Piedras et al., 1998). Active oxygen species may play a role in signalling, leading to changes in gene expression, and possibly also have a direct antimicrobial activity (Hammond-Kosack and Jones, 1997). Phosphatidic acid was rapidly, transiently and specifically produced upon Avr4 treatment of *Cf-4* tobacco cells, mainly generated by phospholipase C and diacylglycerol kinase action. Phosphatidic acid could play a role in triggering responses such as an oxidative burst (De Jong et al., 2004). Two mitogen activated protein kinases (MAPKs) and two related calcium-dependent protein kinases (CDPKs) have been identified that are actively phosphorylated upon Avr9 treatment (Romeis et al., 1999, 2000, 2001). As protein kinases are key signalling components that can be activated in response to extracellular signals, these MAPKs and CDPKs represent active and rapid factors to initiate downstream defence signalling. As for many other resistance proteins, *Cf*-mediated signalling was found to be dependent on the ubiquitin ligase-associated protein SGT1 (Austin et al., 2002; Peart et al., 2002), and recently, a Cf-9 interacting thioredoxin (CITRX) was identified as a negative regulator of Cf-9 but not of Cf-2 (Rivas et al., 2004). Novel *Hcr9*s generated by gene-shuffling and naturally occurring *Hcr9*s were identified that induce an elicitor-independent HR in tobacco species (chapter 5). These auto-activators signal through the *Cf-9* pathway and may be instrumental in elucidating the Cf-9 signal transduction cascade.

Current research is focussed at identifying interacting proteins and downstream components of *Cf* gene-mediated signalling. Several candidate genes were recovered via differential cDNA-AFLP experiments (Durrant et al., 2000; Takken et al., unpublished data), and are currently being tested for their role in *Cf*-dependent HR using Virus-Induced Gene Silencing in *Nicotiana benthamiana* and tomato. Efforts to isolate proteins that are differentially phosphorylated upon *Cf*-mediated signalling are also underway (Stulemeijer et al., unpublished data).

RLP genes in other pathosystems

Many genome and EST sequencing projects have recently been initiated of which the sequences are publicly available. Homology searches using *Cf* gene and protein sequences by BLAST (Basic Local Alignment Search Tool) in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) and at The Institute for Genomic Research (TIGR) (http://tigrblast.tigr.org/tgi/) showed that numerous sequences highly homologous to *Cf* genes (E value < $1e^{-50}$ at the nucleotide level) are present in the genera *Lycopersicon, Solanum* and *Capsicum*. For example, sequences most homologues to the recently identified *OR Hcr9*s have not been identified in *Lycopersicon* species yet, but have been identified in *Solanum tuberosum*, which indicates the existence of ancestral *OR* genes before *Lycopersicon* and *Solanum* speciation. Additionally, BLAST analysis on protein level indicates that RLP genes are abundantly present in many extensively studied plant species. RLP genes have not only been identified in the Solanaceous genera *Lycopersicon, Solanum*, *Nicotiana* and *Capsicum*, but also in plants like Arabidopsis, lettuce (*Lactuca sativa*), apple (*Malus* x *domestica*), *Medicago trunculata*, wheat (*Triticum aestivum*) and rice (*Oryza sativa*). Many of these homologous sequences are expressed, although for the majority a function in disease resistance or another biological function is still speculative. However, for a few (*Ve*, *HcrVf2*, *RPP27* and *LeEix*) a function in microbial recognition and resistance has been reported (Kawchuk et al., 2001; Ron and Avni, 2004; Tör et al., 2004; Vinatzer et al., 2001). Details on the genetic organisation, structure, and mode of action of these RLPs are presented below.

Tomato *Ve* genes against *Verticillium albo-atrum*

Soil-borne fungi of the genus *Verticillium* cause vascular wilt diseases that result in severe yield and quality losses in many crops, including alfalfa, cotton, cucurbits, eggplant, mint, potato, tomato, strawberry and sunflower (Domsch et al., 1980). In a few cases, effective control of *Verticillium* wilt has been reported in specific crops that exhibit race-specific resistance (Lynch et al., 1997; Schaible et al., 1951). In tomato, resistance to race 1 of *Verticillium dahliae* and other species is conferred by a single dominant *Ve* gene that was mapped to linkage group IX (Diwan, et al., 1999; Kawchuk et al., 1994, 1998).

Characterisation of the tomato *Ve* locus identified two closely linked inverted genes, *Ve1* and *Ve2* (Kawchuk et al., 2001). Successful complementation analysis with *Ve1* and *Ve2* in susceptible tomato has not been described yet. However, expression of the individual *Ve* genes in susceptible potato plants conferred resistance to an aggressive race 1 isolate of *Verticillium albo-atrum*. The finding that both *Ve* genes confer resistance in potato to *Verticillium albo-atrum* is surprising as the proteins they encode differ in their C-terminal parts (Kawchuk et al., 2001). It is possible that the Ve1 and Ve2 proteins both recognise the same Avr factor or are involved in recognition of different pathogen-derived ligands. This would require the pathogen to overcome at least two different *Ve* gene specificities. These data also suggest that *V. dahliae* and *V. albo-atrum* share Avr factors.

The deduced primary structures of Ve1 and Ve2 comprise a signal peptide, 38 imperfect LRRs, a membrane-spanning hydrophobic sequence and a C-terminal cytoplasmic endocytosis signal (YXXΦ) (Kawchuk et al., 2001). A PEST-like sequence, which is often involved in ubiquitinisation, internalisation and degradation of proteins (Rogers et al., 1986), resides at the C-terminal domain of Ve2. This suggests that the *Ve* genes encode a class of cell-surface located glycoproteins with receptor-mediated endocytosis-like and PEST signals.

By a candidate gene approach potential *Verticillium dahliae* resistance genes were identified from *Solanum lycoperisicoides* (Chai et al*.,* 2003), *S. tuberosum* (Simko et al., 2004) and scarlet eggplant (*S. aethiopicum*) (Yi and Hou, unpublished data), which are highly homologous to *Ve1*, including the above described domains. The presence of highly homologous genes to *Ve1* in other Solanaceous species may indicate that the *Ve* genes of different species mediate the recognition of conserved pathogen-derived elicitor molecules.

Apple *HcrVf2* against *Venturia inaequalis*

Scab caused by the fungal pathogen *Venturia inaequalis* is the most common disease of cultivated apple (*Malus* x *domestica*). Analogous to the race-specific Avr proteins from *C. fulvum* (Joosten and De Wit, 1999), *V. inaequalis* also appears to secrete race-specific proteins that induce an HR in a resistant apple variety (Win et al., 2003). *Vf* resistance has been introgressed from the small-fruited wild apple species *Malus floribunda* 821. Two independent map-based cloning efforts identified comparable clusters of receptor-like genes with homology to the *Cf* resistance gene family (Vinatzer et al., 2001; Xu and Korban, 2002). The first two of the four *HcrVf* genes (Homologue of the *C. fulvum* resistance genes of the *Vf* region) in both clusters are identical. Although only parts of the *Vf* locus have been sequenced, the intergenic regions between the *HcrVf* genes appear larger than in the *Cf* clusters. However, the *HcrVf* and *Cf* clusters share the structure of tandemly repeated genes, which suggests a similar mode of evolution. *HcrVf1*, *HcrVf2* and *HcrVf4* are expressed both in the presence or absence of the pathogen, and *HcrVf3* may be a pseudo gene or may have a very low transcription level (Vinatzer et al., 2001). By transgenic complementation analysis in a susceptible apple cultivar, *HcrVf2* was recently shown to be the functional *R* gene against *V. inaequalis* (Belfanti et al., 2004). A recent evolutionary analysis of the *Vf* gene family

suggested that the *HcrVf* genes evolved mainly by divergent somatic variation (Xu and Korban, 2004), as no sequence exchange between the four homologues was detected. Two sequential duplication events might have generated the four *Vf* genes from a single *Vf* progenitor gene. However, the evolutionary model proposed for the *HcrVf* genes is based on only a single *HcrVf* gene cluster. Isolation of additional *HcrVf* loci can shed more light on *HcrVf* gene evolution.

Comparison of the deduced amino acid sequence of HcrVf and Hcr9 proteins clearly shows the same type of overall protein structure with similar domains. Additionally, the level of conservation and the variability of the different domains between the members of each of the two families is similar, with domains B and the N-terminal part of domain C being most variable.

Arabidopsis *RPP27* against *Peronospora parasitica*

Peronospora parasitica (*Peronosporaceae* family) is a naturally occurring oomycete parasite of Arabidopsis and the causal agent of downy mildew disease. Members of *Peronosporaceae* are obligatory biotrophic plant pathogens and cause significant damage to many crop species (Channon, 1981). Parasitism in this group involves the development of hyphal networks within host tissue and the production of haustoria that invade host cells. Haustoria penetrate plant cell walls and invaginate the host cell plasma membrane, which is accompanied by stimulated production of additional host plasmalemma (Sargent, 1981).

Resistance to *P. parasitica* is mediated by numerous *RPP* resistance genes, which are all members of the intracellular CC-NBS-LRR and TIR-NBS-LRR gene families (reviewed by Holub, 2001). The *RPP27* gene of the Arabidopsis accession Ler-0, however, encodes an RLP (Tör et al., 2004). This *Cf*-like gene was used to complement susceptible Col-0 plants. Progeny from these plants were resistant to at least five Col-compatible *P. parasitica* isolates. In the Col-0 plants, a susceptible allele of *RPP27* was identified that differs from *RPP27* by three nucleotides and a deletion of 68 nucleotides in the open reading frame. As this deletion causes a frameshift and leads to a premature stop codon, the *RPP27* allele of Col-0 is a pseudogene. Interestingly, these results indicate that resistance in Arabidopsis against *P. parasitica* is conferred by different types of *R* genes.

Tomato *LeEix* against *Trichoderma*

Besides recognition of specific pathogen-derived Avr proteins, plants also respond to proteins produced by non-pathogenic micro-organisms. *Trichoderma* species are opportunistic, avirulent plant symbionts and mycoparasites (Whipps, 2001). *T. viride* does not only attack root-colonising fungi but also penetrates the host plants. The *T. viride* ethylene-inducing xylanase (EIX) elicits defence responses in host plants and thereby combats fungal pathogens indirectly. In both tomato and tobacco EIX recognition is controlled by a single dominant locus (Bailey et al., 1993; Ron et al., 2000) and is independent of the xylanase enzyme activity (Enkerli et al., 1999; Furman-Matarasso et al., 1999). The EIX locus in tomato

comprises three homologous *LeEix* genes of which two have been cloned (Ron and Avni, 2004). *LeEix1* and *LeEix2* encode Cf-like proteins. Specific for the LeEix proteins is the presence of leucine-zipper motif in the B domain and 31 imperfect LRRs. In common with the Cf proteins is the presence of the YXXΦ endocytosis signal in the C-terminal cytoplasmic tail. Both LeEix1 and LeEix2 bind EIX, but only LeEix2 could transmit the signal that induces HR. Mutations in the endocytosis signal in *LeEix2* abolished its ability to induce the HR, suggesting that endocytosis plays a key role in the *LeEix* signal transduction pathway (Ron and Avni, 2004).

EIX also interacts with a cytoplasmic small ubiquitin-related modifier protein (SUMO), showing that EIX should be internalised (Hanania et al., 1999). Sumolyation of proteins may influence the targeting of substrate proteins to specific cellular compartments or structures (Seeler and Dejean, 2003). Therefore, SUMO may play a role in the repression of defence responses and programmed cell death (Hanania et al., 1999). This repression is lifted upon EIX treatment by translocation of proteins involved in the repression (Hanania et al., 1999). It is proposed that the binding of the EIX elicitor to the LeEix2 protein causes a ligand-induced conformational change spreading from the extracellular to the cytoplasmic domain (Ron and Avni, 2004). Alternatively, the binding of EIX to LeEix2 may induce receptor-mediated endocytosis, thus allowing the receptor and/or EIX to interact with a cytoplasmic protein and hence generate a signal to induce the defence response.

By gene-specific silencing it was shown that in tobacco EIX perception is mediated by a gene that is highly homologous to *LeEix* (Ron and Avni, 2004), providing the first example in which almost identical genes from different species are involved in the recognition of the same elicitor.

Concluding remarks

In general, the RLP genes confer resistance to pathogens that grow extracellularly. The proposed extracellular LRRs and plasma-membrane localisation of RLP proteins are consistent with the extracellular location of pathogen-derived elicitors. For many of the tomato *Cf* genes, the corresponding elicitor molecules of *C. fulvum* have been identified. However, few biochemical data on elicitor perception have been described. Besides the PEST- and endocytosis signals present in some RLPs, these receptor-like proteins lack distinct signalling domains. Several downstream signal transduction components have been identified, but it is still unclear how the extracellular elicitor perception and the cytoplasmic signal transduction cascade are interconnected. Studies investigating *Cf*-mediated elicitor perception and signal transduction pathways may benefit from the recent study on the EIX/LeEix interaction (Avni and Ron, 2004). The RLKs FLS2 (Gomez-Gomez and Boller, 2000) and Xa21 (Song et al., 1995) are known to function as R proteins. However, most RLKs described so far are involved in many developmental and symbiotic processes

(reviewed by Diévart and Clark, 2004). Similar to the CLAVATA complex, which contains the RLK CLV1 and the Cf-like protein CLV2 (Torii, 2000), the Cf-like resistance proteins may form a complex with an RLK to enable elicitor perception and initiation of downstream defence responses (Joosten and De Wit, 1999).

The matching elicitors of the RLP genes *Ve*, *HcrVf2*, *RPP27* have not been identified yet. However, sequence information of these recently identified resistance genes was rapidly exploited in linkage disequilibrium mapping through candidate gene approaches and has resulted in additional knowledge on conservation and variation in homologous sequences (Chai et al., 2003; Simko et al., 2004; Yi and Hou, unpublished data). In the Arabidopsis genome approximately 60 *Cf*-like genes have been identified (Shiu and Bleecker, 2003). Together, these data indicate that RLP genes are widespread in the plant kingdom. Some of these RLPs may indeed be involved in resistance against unknown plant pathogens (Cohn et al., 2001; Jones and Takemoto, 2004). *RPP27* is the first RLP gene implicated in pathogen resistance in Arabidopsis, enabling the use of Arabidopsis techniques to investigate the mechanisms of RLP function.

A challenge for the near future is to investigate which RLPs are mainly involved in recognising and rejecting pathogens and which play an important role in developmental processes. Most RLP resistance genes are located in clusters and are highly conserved, yet each RLP gene appears to mediate recognition of a single distinct elicitor. Detailed evolutionary studies have mainly focussed on the tomato *Cf* genes, and future studies on evolution of other RLP resistance genes will reveal whether they have evolved by similar mechanisms. Identification and functional analysis of novel RLP genes derived from different plants will reveal which sequence and structure variation is tolerated and which characteristics of RLPs remained conserved during evolution and speciation.

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Summary

As the world population continues to grow, it is vital to improve crop yields. Therefore, adequate protection of crop plants against pathogens is important. Efforts to protect agriculturally important crop plants against their pathogens may benefit from knowledge of resistance genes (*R* genes) in natural plant populations.

In **chapter 1** the different ways by which plant resist pathogens are discussed. Several classes of *R* genes have been identified that function according to the gene-for-gene relationship, by which for each *R* gene in the plant a complementary avirulence (*Avr*) gene is present in the pathogen. Two models have been proposed that describe the evolutionary dynamics of *R* genes in natural plant populations. The birth-and-death model assumes that novel *R* genes are continuously generated ('birth'), which may be rapidly overcome by virulent pathogen races, and subsequently lost from the plant population ('death'). In contrast, the trench warfare model describes that plants maintain *R* genes over long periods of time, although the frequency of individual *R* genes may fluctuate.

The *Cladosporium fulvum*-tomato pathosystem is an ideal gene-for-gene model system for studying the interaction between biotrophic pathogenic fungi and plants. *C. fulvum* secretes Avr proteins upon infection of a tomato leaf. These Avrs are specifically recognised by tomato plants with matching *C. fulvum* resistance genes (*Cf* genes), leading to induction of defence reponses and arrest of fungal growth. *Cf* genes encode membrane bound receptor-like proteins (RLPs), consistent with the predicted extracellular perception of the Avr proteins. The *Avr4-Cf-4* and *Avr9-Cf-9* gene pairs have been studied extensively. The *Cf-4* and *Cf-9* genes are members of the Homologues of *C. fulvum* resistance gene *Cf-9* (*Hcr9*) gene family, which not only contains *Cf* genes, but also genes without a known function in *C. fulvum* resistance. Most *Hcr9*s reside in clusters of tandemly repeated homologues, which allows extensive sequence exchange between *Hcr9*s. This sequence exchange provides generation of novel *Hcr9*s, which potentially confer resistance to *C. fulvum*.

The evolution of the tomato *Hcr9* gene family has been described by the isolation of *Hcr9* clusters from a few genotypes. However, variation in *Hcr9*s that mediate responsiveness to the same elicitor has not been described, and is the main subject of this thesis. We first studied variation in the *Cf-9* gene in the wild tomato species *Lycopersicon pimpinellifolium*, from which *Cf-9* originates. In this study, we discovered the *9DC* gene, which also confers Avr9 recognition (**chapter 2**). Avr9 recognition was found throughout the *L. pimpinellifolium* population, and is likely only mediated by the *9DC* and *Cf-9* genes. The *Cf-9* cluster consists of five homologues (*Hcr9-9A*-*Hcr9-9E*), of which *Hcr9-9C* is the *Cf-9* gene. The first half of *9DC* is nearly identical to the *Hcr9-9D* gene, a homologue adjacent to *Cf-9*, and the second half is nearly identical to that of *Cf-9*. *9DC* occurs at a higher frequency in the *L. pimpinellifolium* population than *Cf-9* and is more widely spread. These data suggested that *Cf-9* evolved from *9DC* by intragenic recombination between *9DC* and another *Hcr9*. 9DC has the same activity and specificity as Cf-9, although it differs by 61 amino acids. Thus, natural Hcr9 proteins with the same function in resistance may vary significantly.

We further studied the close evolutionary relationship between *Cf-9* and *9DC* by the isolation of the complete *9DC* cluster from *L. pimpinellifolium* LA1301 (**chapter 3**), from which *9DC* was isolated. A phage library was screened for phages containing *9DC*, and *9DC*-containing clones were assembled into a contig and sequenced. The *9DC* cluster contains three *9DC* genes (*9DC1*, *9DC2* and *9DC3*), orthologues of the *Hcr9-9A* and *Hcr9-9E* genes

from the *Cf-9* cluster, as well as three *Hcr9* fragments. Numerous rearrangements in the central region of the *9DC* cluster, but not at the ends, were identified by comparing the *Cf-9* and *9DC* gene clusters. Furthermore, this comparison provided strong indications that *Cf-9* is ancestral to *9DC*, in contrast to what was suggested in chapter 2. Multiple unequal inter- and intragenic recombination events have resulted in the three *9DC* genes. All three *9DC* genes confer Avr9 recognition in agroinfiltration overexpression assays in tobacco. However, only *9DC2* is active upon agroinfiltration under control of its native promoter. Therefore, *9DC2* is most likely the main determinant of Avr9 recognition in *L. pimpinellifolium* LA1301.

The low variation in *Cf-9* and *9DC* in the *L. pimpinellifolium* population suggests that *Cf* genes might be highly conserved as a result of selection pressure. However, the complex structure of the *Hcr9* gene family could also allow convergent evolution of *Cf* genes with the same specificity. To address the question how *Hcr9*s that recognise the same *C. fulvum* elicitor have evolved, we studied the variation in functional homologues of *Cf-4* and *Cf-9* in wild tomato species (**chapter 4**). Avr4- and Avr9-responsive plants were identified throughout the *Lycopersicon* genus. *Hcr9*s from the responding *Lycopersicon* plants were amplified by PCR and independent *Hcr9*s were tested for conferring Avr4 or Avr9 responsiveness by agroinfiltration overexpression assays in tobacco. The isolation of highly conserved functional homologues of *Cf-4* and *Cf-9* from diverged tomato species strongly suggests that these genes are ancient and predate *Lycopersicon* speciation. Moreover, these results suggest that *C. fulvum* is an ancient pathogen of the *Lycopersicon* genus and has imposed selection pressure on *Lycopersicon* plants, resulting in the maintenance of functional homologues of *Cf-4* and *Cf-9* in different wild tomato species.

In our screen for functional homologues of *Cf-4* and *Cf-9*, we also identified two *Hcr9*s from *L. peruvianum*, which give an elicitor-independent HR upon transient overexpression by agroinfiltration in several tobacco species, and were designated auto-activators of HR (**chapter 5**). Additional auto-activators were generated by *in vitro* shuffling of *Hcr9* sequences. The auto-activators exhibited different activities in five selected tobacco species, and were shown to function in the same signalling pathway as *Cf-9*. Several models which explain the elicitor-independency of the auto-activators and their potential use in unravelling the mechanisms of *Cf* gene-mediated resistance are discussed.

The general discussion (**chapter 6**) gives an overview on the current status of the research on RLP disease resistance genes in plants, including the results described in the previous chapters. The genetic mechanisms and dynamics of evolution of RLP disease resistance genes are described, as well as the variation and evolution of *Cf* genes in wild tomato populations. The pathosystems from which RLP disease resistance genes have been isolated are also briefly described. The current status of the research on elicitor perception and signal transduction mediated by RLP genes is discussed, with emphasis on the extensively studied tomato *Cf* genes.

Samenvatting

De wereldbevolking zal de komende jaren sterk toenemen, waardoor het van cruciaal belang is om de agrarische productie te verhogen. Inspanningen om belangrijke voedselgewassen tegen hun pathogenen te beschermen kunnen profiteren van kennis van resistentiegenen (*R* genen) in natuurlijke populaties planten.

In **hoofdstuk 1** worden de verschillende soorten resistentie van planten tegen pathogenen besproken. Verschillende klassen *R* genen zijn geïdentificeerd die functioneren in overeenstemming met de gen-om-gen relatie, waarin voor elk *R* gen in de plant een complementair avirulentie (*Avr*) gen aanwezig is in het pathogeen. In twee modellen wordt de de evolutionare dynamiek van *R* genen in natuurlijke planten populaties beschreven. Het birth-and-death model veronderstelt dat nieuwe *R* genen continue worden aangemaakt ('birth'), welke snel doorbroken worden door virulente stammen van het pathogeen, en als gevolg daarvan uit de populatie zullen verdwijnen ('death'). In het trench warfare model worden *R* genen gedurende een lange periode in de plant gehandhaaft, en fluctueert de frequentie van individuele *R* genen in de tijd.

Het *Cladosporium fulvum*-tomaat pathosysteem volgt de gen-om-gen relatie, en is een modelsysteem om de interactie tussen biotrofe pathogene schimmels en planten te bestuderen. *C. fulvum* scheidt tijdens infectie van een tomatenplant Avr eiwitten uit, welke specifiek herkend worden in tomatenplanten die de complementaire *C. fulvum* resistentie genen (*Cf* genen) bevatten. Deze herkenning leidt tot inductie van afweerreacties waardoor geen verdere schimmelgroei mogelijk is. *Cf* genen coderen voor membraan-gebonden receptor-achtige eiwitten, wat overeenkomt met de voorspelde extracellulaire herkenning van de Avr eiwitten. De *Avr4-Cf-4* en *Avr9-Cf-9* genparen zijn uitvoerig bestudeerd. De *Cf-4* en *Cf-9* genen behoren tot de *Hcr9* (Homologen van het *C. fulvum* resistentie gen *Cf-9*) genfamilie, waar zowel *Cf* genen als *Hcr9*s met onbekende functie toe behoren. De meeste *Hcr9*s zijn gelokaliseerd in clusters van opeenvolgende homologen, wat kan leiden tot uitwisseling van sequenties tussen *Hcr9*s. Middels deze sequentie uitwisseling kunnen nieuwe *Hcr9*s, en daarmee mogelijk nieuwe *Cf* genen ontstaan.

De evolutie van de *Hcr9* genfamilie is gekarakteriseerd door analyse van *Hcr9* clusters uit enkele tomaten genotypes. De variatie in *Hcr9*s die betrokken zijn bij de herkenning van dezelfde elicitor is echter nog niet beschreven, en vormt het belangrijkste onderwerp van dit proefschrift. De variatie in het *Cf-9* gen in de wilde tomatensoort *Lycopersicon pimpinellifolium*, waaruit *Cf-9* afkomstig is, is eerst bestudeerd. In deze studie is het *9DC* gen geïdentificeerd, dat dezelfde functie in Avr9 herkenning heeft als *Cf-9* (**hoofdstuk 2**). Avr9 herkenning werd verspreid over de hele *L. pimpinellifolium* populatie gevonden, en waarschijnlijk zijn alleen de *9DC* en *Cf-9* genen hier verantwoordelijk voor. Het *Cf-9* cluster betsaat uit vijf homologen (*Hcr9-9A-Hcr9-9E*), waarvan *Hcr9-9C* het *Cf-9* gen is. De eerste helft van *9DC* is bijna identiek aan *Hcr9-9D*, een homoloog naast *Cf-9*, en de tweede helft is bijna identiek aan *Cf-9*. De frequentie van *9DC* in de *L. pimpinellifolium* populatie is hoger dan die van *Cf-9*, en *9DC* wordt gevonden in een groter geografisch gebied. Dit suggereerde dat *Cf-9* geëvolueerd is uit *9DC* door intragene recombinatie tuseen *9DC* en een andere *Hcr9*. Het 9DC eiwit heeft dezelfde activiteit en specificiteit als Cf-9, hoewel beide genen 61 aminozuren verschillen. Dit toont aan dat natuurlijke Hcr9 eiwitten met dezelfde functie aanzienlijk kunnen verschillen.

De evolutionaire relatie tussen *Cf-9* en *9DC* is verder bestudeerd door het *9DC* cluster te isoleren uit *L. pimpinellifolium* LA1301 (**hoofdstuk 3**), waaruit *9DC* afkomstig is.

Uit een faagbank zijn kloons geïsoleerd die *9DC* bevatten. Deze kloons zijn samengevoegd tot een ononderbroken sequentie, waarna het *9DC* cluster volledig is gesequenced. Het *9DC* cluster bevat drie *9DC* genen (*9DC1*, *9DC2* en *9DC3*), orthologen van de *Hcr9-9A* en *Hcr9-9E* genen van het *Cf-9* cluster, en drie fragmenten van *Hcr9*s. Door vergelijking van de *Cf-9* en *9DC* clusters zijn meerdere veranderingen in het centrale gedeelte van het *9DC* cluster geïdentificeerd, maar geen aan de uiteinden van het cluster. Bovendien zijn sterke aanwijzingen gevonden dat *9DC* het product is van recombinatie tussen *Cf-9* en *Hcr9-9D*, in tegenstelling tot wat in hoofdstuk 2 werd gesuggereerd. De drie *9DC* genen zijn het resultaat van meerdere inter- en intragene recombinaties. Alledrie de *9DC* genen zijn actief in Avr9 herkenning wanneer ze tot overexpressie worden gebracht in een agroinfiltratie experiment, maar achter hun eigen promoter is alleen *9DC2* actief. Waarschijnlijk is *9DC2* dus het belangrijkste *9DC* gen dat betrokken is bij de Avr9 herkenning in *L. pimpinellifolium* LA1301.

De geringe variatie in *Cf-9* en *9DC* in de *L. pimpinellifolium* population doet vermoeden dat *Cf* genen als gevolg van een selectiedruk sterk geconserveerd zijn. Door de complexe structuur van de *Hcr9* genfamilie zouden echter ook door convergente evolutie *Cf* genen met dezelfde specificiteit kunnen ontstaan. Om te bestuderen hoe *Hcr9*s die dezelfde *C. fulvum* elicitor herkennen evolueren, is de variatie in functionele homologen van *Cf-4* en *Cf-9* in wilde tomatensoorten onderzocht (**hoofdstuk 4**). In het gehele *Lycopersicon* genus zijn planten geïdentificeerd die Avr4 of Avr9 herkennen. Van deze planten zijn middels PCR *Hcr9*s geamplificeerd, waarvan vervolgens middels agroinfiltratie overexpressie proeven in tabak getest is of deze betrokken zijn bij Avr4 of Avr9 herkenning. De isolatie van een aantal sterk geconserveerde functionele homologen van *Cf-4* en *Cf-9* uit uiteenlopende tomatensoorten suggereert sterk dat deze genen oud zijn en al bestonden voor de *Lycopersicon* soortsvorming. Deze resultaten suggereren bovendien dat *C. fulvum* een oud pathogeen van het *Lycopersicon* genus is, en tijdens de evolutie van *Lycopersicon* selectiedruk heeft uitgeoefent, waardoor de functionele homologen van *Cf-4* en *Cf-9* in verschillende wilde tomatensoorten bewaard zijn gebleven.

Tijdens de studie naar functionele *Cf-4* en *Cf-9* homologen zijn ook twee *Hcr9*s uit *L. peruvianum* geïsoleerd die een elicitor-onafhankelijke overgevoeligheidsreactie (hypersensitive response of HR) induceren in verschillende tabakssoorten wanneer ze middels agroinfiltratie tot overexpressie worden gebracht. Deze *Hcr9*s zijn auto-activatoren van HR genoemd (**hoofdstuk 5**). Daarnaast zijn tevens door *in vitro Hcr9* sequenties uit te wisselen auto-activatoren gegenereerd. De auto-activatoren vertonen verschillende activiteiten in vijf geselecteerde tabakssoorten, en gebruiken dezelfde signaal transductie cascade als *Cf-9*. Een aantal modellen die de elicitor-onafhankelijkheid van de auto-activatoren verklaren en de bruikbaarheid van de auto-activatoren in het bestuderen van de mechanismen van de resistentie door *Cf* genen worden besproken.

In de algemene discussie (**hoofdstuk 6**) wordt een overzicht van de huidige status van het onderzoek aan *Cf*-achtige *R* genen in planten gegeven, waaronder de resultaten van de vorige hoofdstukken. De genetische mechanismen betrokken bij en de evolutionare dynamiek van *Cf*-achtige *R* genen wordt besproken, alsmede de variatie en evolutie van *Cf* genen in wilde tomaten populaties. De pathosystemen waaruit *Cf*-achtige *R* genen zijn geïsoleerd worden ook kort besproken. De stand van zaken van het onderzoek aan elicitor perceptie en signaal transductie van *Cf*-achtige genen wordt besproken, met nadruk op de uitvoerig bestudeerde *Cf* genen uit tomaat.

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Marco

Curriculum vitae

Marco Kruijt is geboren op 26 mei 1976 te Zoetermeer. In 1994 behaalde hij het VWO diploma aan de J.C. de Glopper te Capelle aan de IJssel. Hetzelfde jaar is hij begonnen aan de studie Bioprocestechnologie aan de Wageningen Universiteit, waar hij heeft gekozen voor de specialisatie Plantenceltechnologie. Zijn eerste afstudeervak werd uitgevoerd bij het Laboratorium voor Moleculaire Biologie van de Wageningen Universiteit. Hier heeft hij onderzoek gedaan naar de betrokkenheid van een wortel receptor kinase bij de regulering van de vorming van stikstofbindende wortelknolletjes in erwt door *Rhizobium* bacteriën. Daarna liep hij stage bij het Centrum Terrestrische Oecologie van het Nederlands Instituut voor Oecologisch Onderzoek in Heteren. Met materiaal verzameld op de Krakatau eilanden in Indonesië is onderzoek gedaan naar mogelijke hybridisatie tussen verschillende *Ficus* soorten op deze eilanden. Zijn tweede afstudeervak heeft hij uitgevoerd bij het Laboratorium voor Fytopathologie van de Wageningen Universiteit, waarbij hij met name variatie in het *Cladosporium fulvum* resistentiegen *Cf-9* in de wilde tomaat *Lycopersicon pimpinellifolium* heeft onderzocht. Zijn studie aan de Wageningen Universiteit werd in september 1999 afgerond. Op 1 november 1999 is hij begonnen aan een promotieonderzoek bij het Laboratorium van Fytopathologie, onder begeleiding van Prof. dr. ir P.J.G.M. De Wit en Dr. ir. B.F. Brandwagt. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

Training and supervision plan of the Graduate School Experimental Plant Sciences

1. Participation in postgraduate courses and workshops:
   a) EPS Autumn school "Interactions between plants and attacking organisms" (2000).
   b) EPS course "Guide to digital scientific artwork" (2001).
   c) EPS Autumn school "Disease resistance in plants" (2002).
   d) EPS course "Molecular phylogenies: reconstruction and interpretation" (2003).

2. Participation in international meetings, including oral and poster presentations:
   a) 9th International congress on molecular plant-microbe interactions, Amsterdam, The Netherlands (1998), attendance.
   b) 6th International congress of plant molecular biology, Quebec, Canada (2000), poster presentation.
   c) Symposium Durable disease resistance, Ede, The Netherlands (2000), poster presentation.
   d) 8th Congress of the European society for evolutionary biology, Arhus, Denmark (2001), oral and poster presentation.
   e) Plant genetics 2003: Mechanisms of genetic variation, Utah, United States (2003), oral and poster presentation.

3. Participation in national meetings, including oral and poster presentations:
   a) Annual EPS PhD students day (1999-2003), poster presentations.
   b) Annual ALW (Earth and Life Sciences) meetings (2000-2004), poster and oral presentations.
   c) Annual EPS theme symposia (2000, 2002, 2003).
   d) EPS flying and other seminars (2000-2003).

4. Organisation:
   a) Organisation of annual EPS PhD students day 2001.

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