Recognition of the *Cladosporium fulvum* Ecp2 elicitor in tomato and non-host plants.

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General introduction and outline

PLANT-PATHOGEN INTERACTIONS

Introduction

Plants are the basis of all the food on which humans, animals, and other heterotrophs depend. They grow and produce well if the environmental conditions are favourable. Plants may also get diseased that causes crop losses and reduction in quality. Plants are continuously exposed to a wide range of pathogens. Plant pathogens are found in different kingdoms and include viroids, viruses, bacteria, mycoplasma, fungi, oomycetes, nematodes, insects and animals (Agrios, 1997). Despite being constantly challenged by aspiring pathogens, disease is generally rare. Pathogens have a limited range of host plants on which they cause disease. Often only plants of a single genus are host for a certain pathogen. Subsequently, in this single genus, plants can have specific 'host' resistance to combat these 'host pathogens'. All other plants are by definition 'nonhost plants', and the attacking organisms are 'nonhost pathogens'. Nonhost resistance is supposed to be highly effective and durable.

The challenging road to infection *versus* multiple strategies for resistance

For the identification mechanisms that lead to host- and nonhost resistance, it is important to know the obstacles a pathogen encounters during its interaction with the plant. Roughly, there are five different obstacles that a plant pathogen must overcome before it succeeds in successful infection (Figure 1). These can include preformed structural barriers or chemical compounds and various induced defence responses (Thordal-Christensen, 2003).

(*i*) Pathogens require signals from the plant to induce cell differentiation and express essential pathogenicity genes. This requirement for cues from the plant is obvious. In rust fungi hyphal differentiation for example is induced by the surface topography of the plant (Hoch, *et al*., 1987). Additionally, it is suggested that the composition of the surface wax triggers the activation of the development of a differentiated appressorium of barley powdery mildew fungus (Tsuba *et al*., 2002).

(*ii*) Presence of pre-formed barriers like plant cell walls, antimicrobial enzymes and secondary metabolites are early obstacles that are difficult to take by the pathogen. These barriers are important in defence against many host- and non-host pathogens, but their success in preventing successful infection probably depends on the degree of co-evolution of the pathogen and the host (Thordal-Christensen, 2003). Adaptation of the oat root pathogen *Gaeumannomyces graminis* var. *avenae* to the antimicrobial compound

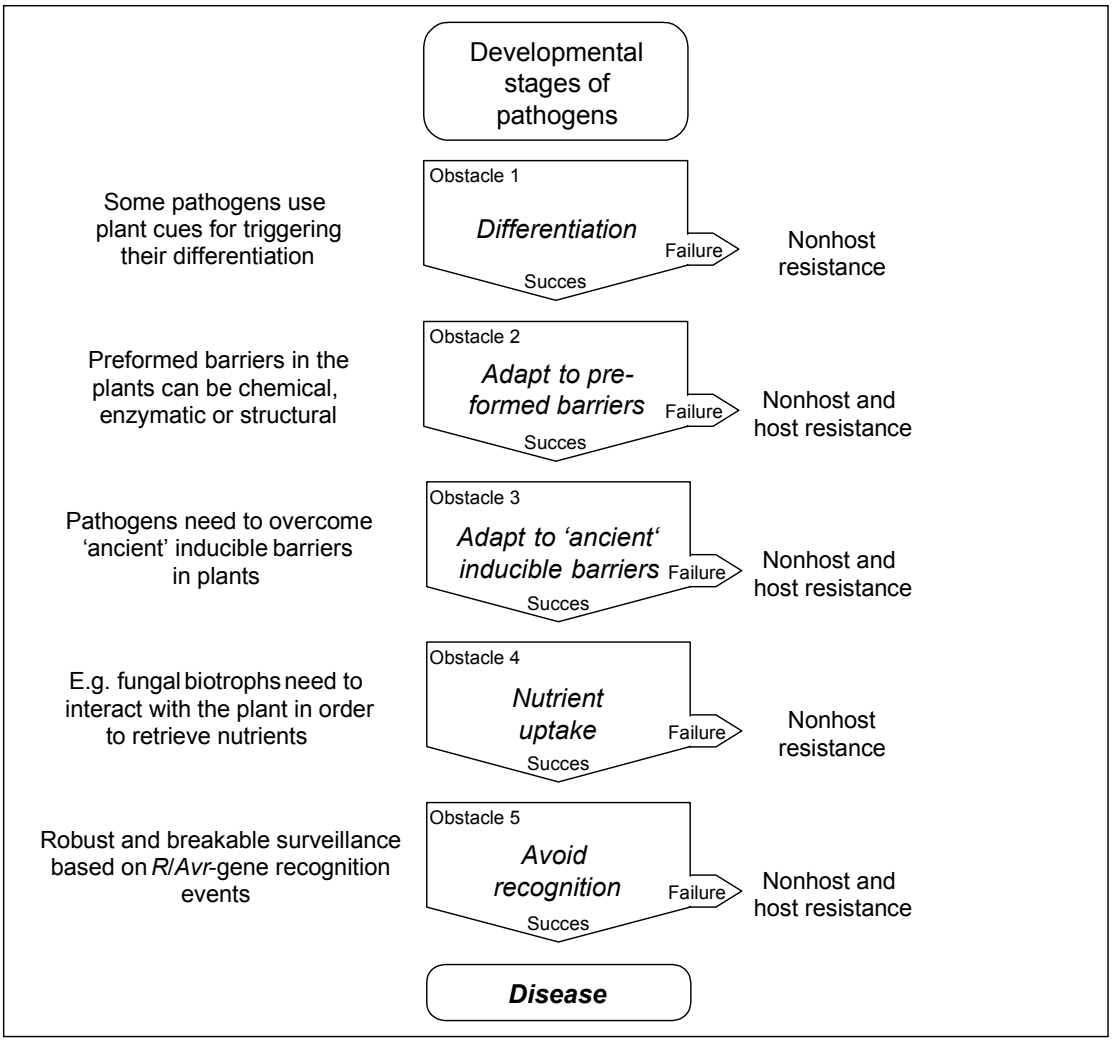

Figure 1. A tentative view of barriers which a pathogen encounters when it attempts to cause disease. Stages at which nonhost and host resistance can been manifested are indicated (after Thordal-Christensen, 2003).

avenacin is mediated by detoxification. The closely related *G. graminis* var. *tritici*, however, is a nonhost pathogen of oats because it lacks the enzyme that detoxifies avenacin (Papadopoulou *et al*., 1999). (*iii*) 'General elicitors' are present or may be released during the invasion of both host pathogens and nonhost pathogens, and the barriers that are activated in response to these elicitors contribute to resistance towards both types of pathogens. Flagellin, a protein of the bacterial flagella, serves as such an elicitor (Felix *et al*., 1999). Other general elicitors are released during pathogenesis and are often indispensable for the pathogen. Plants are capable te recognise them in the same way as animals recognise 'pathogen-associated molecular patterns' (PAMPs) (Parker, 2003). The use of general elicitors together with the involvement of LRR-kinases and MAP kinase cascades make this defence mechanism reminiscent of animal 'innate immunity' system

(Cohn *et al.*, 2001; Gómez-Gómez *et al*., 2002; Jones and Takemoto, 2004). Other examples of defence mechanisms that may have similarities to innate immunity are the activation of defence responses in the nonhost plants parsley and potato by Pep-13, a peptide fragment from *Phytophthera sojae* (Nürnberger *et al.*, 1994; Nennstiel *et al.*, 1998; Brunner, *et al.*, 2002) and the activation of defences in nonhost tobacco by the 'harpin' protein of *Pseudomonas syringae* (Lee *at al*., 2001).

(*iv*) Upon entering the host tissue, many pathogens develop sophisticated means to acquire nutrients from the host. Rust and powdery mildew fungi, for instance, initiate a biotrophic interaction with their host by haustoria that facilitate the transport of nutrients across specialised membranes (Staples, 2001; Mendgen and Hanh, 2002). As these interactions may be very specific, it is reasonable to expect that the co-evolution of pathogens with their host plant species has resulted in pathogens being adapted to a restricted host range.

(*v*) The last obstacle faced by the pathogen is based on classical gene-for-gene interactions. Oort (1944), and Flor (1942, 1946) independently proposed the gene-for-gene concept to explain host resistance using the *Ustilago tritici* - wheat and the *Melampsora lini* - flax interactions, respectively. The observed resistance was often genotype-specific and generally depended on a monogenic, dominant trait. In addition, avirulence of the pathogen was dependent of a monogenic, dominant trait as well. The gene-for-gene concept consequently postulates that resistance is based on specific recognition of a product of an avirulence (*Avr*) gene of the pathogen by the product of a corresponding resistance (*R*) gene of the plant. Effectors or elicitors are often pathogen proteins encoded by *Avr* genes that probably evolved to subvert host processes for promotion of the pathogen life cycle but simultaneously may act as avirulence factors. The known elicitors show hardly any sequence similarity and a beneficial function for the pathogen has been demonstrated for only a few (Gabriel, 1999; White *et al*., 2000; Kjemtrup *et al*., 2000; Van 't Slot and Knogge, 2002).

An accumulating number of reports suggests that nonhost pathogens are often rejected because of several simultaneous, but independent recognition events. *Xanthomonas campestris* and *Pseudomonas syringae* pathovars, for instance, express numerous nonhost *Avr* genes, each of which allows the recognition of these pathogens in nonhost plants according to the gene-for-gene model (Whalen *et al*., 1988; Kobayashi *et al*., 1989). Such nonhost *Avr* genes may also exist in fungal and oomycete pathogens. Genetic crosses between pathogenic types of *Magnaporthe grisea* that infect wheat, oat or foxtail millet, showed that single *Avr*-genes account for the host-species specificities of this fungus (Murakami *et al*., 2003; Takabayashi *et al*., 2002). The Ecp2 virulence protein of the tomato pathogen *Cladosporium fulvum* is able to activate the hypersensitive response

(HR) in nonhost *Nicotiana paniculata* (Laugé *et al*., 2000). Various members of the family of elicitin-like proteins of *Phytophthora infestans* and several novel *P. infestans* proteins that are unrelated to elicitins, have been shown to trigger defence-like symptoms in *Nicotiana* species (Kamoun *et al*., 1998; Kamoun, 2001; Fellbrich *et al*., 2002; Qutob *et al*., 2002). These plant responses certainly prefent pathogen proliferation in a quantitative manner, but their relative contribution to nonhost resistance is not entirely clear.

In general, recognition of a pathogen by the host is not required when a pathogen fails to overcome the 1st, 2nd, and 4th obstacles (Figure 1). In addition, recognition of general elicitors may have a crucial role in nonhost resistance. Finally, different pathogens and plant species may have functional homologues of a particular *Avr* - *R* gene pair (Kawchuk *et al*., 2001;Milligan *et al*., 1998; Rossi *et al*., 1998; Van der Vossen *et al*., 2000). This suggests that the same *R* gene may interact with different host and nonhost pathogens.

Structure and function of resistance proteins

Most attention in the research on plant-pathogen interactions has been paid to the resistance genes involved in HR, a localised form of programmed cell death limiting pathogen spread, further reinforcement of the cell walls, and production of antimicrobial compounds such as defence proteins and phytoalexins. The majority of R 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 various plant species that 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 mainly comprises the tomato Cf proteins, which have an extracellular LRR domain, a single transmembrane (TM) domain, and a small cytoplasmic tail (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 extracellular LRR 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 cannot be classified (Martin *et al*., 2003). Most likely, they 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).

In many cases, recognition of the elicitor activates a cascade of reactions in the infected and surrounding cells. Some signalling components are shared by many *R* gene pathways whereas others appear to be pathway-specific (reviewed by Kunkel and Brooks, 2002; Martin *et al*., 2003; and Nimchuk *et al*., 2003). The recognition results in several early responses including rapid ion fluxes, activation of kinase cascades and the generation of reactive oxygen species. These early defence responses are followed by other defence responses that are mediated by two major pathways in defence signalling, one is salicylic acid (SA)-dependent, the other is SA-independent but involves jasmonic acid and ethylene. Transduction of recognition probably requires regulated protein degradation and results in massive changes in cellular homeostasis, including induction of HR.

Evolution of resistance genes

Mutant pathogens that have changed from avirulent to virulent, have a selective advantage as they can grow on host plants that harbour the corresponding *R* gene. These pathogens therefore have a higher change for reproduction. However, in addition to the preformed barriers, plants have a wide range of recognition specificities and as a result susceptibility is therefore the exception. This suggests that co-evolution between host and pathogen frequently occurs in nature. During evolution, new resistance specificities must have been generated to counteract with the newly evolved virulent strains of pathogens.

There are several mechanisms by which sequence diversification of plant resistance genes is promoted, is determined by the genomic context of the *R* genes. Some *R* genes, such as *Hm1* and *RPM1* (Johal and Briggs, 1992; Grant *et al*., 1995) are only present as a single copy and are absent in susceptible plants. The fast majority of *R* genes, however, are organised in complex loci that contain tandemly repeated homologous genes. The tandem array organisation of homologous sequences probably facilitates inter- and intragenic recombination events, unequal crossing over and gene duplications (Hammond-Kosack and Jones, 1996; Michelmore and Meyers, 1998). For example, a detailed analysis of the *Cf* clusters revealed that extensive sequence exchange has occurred between the individual homologues and between individual clusters (Parniske *et al*., 1997, 1999a,b). In addition to events that are sufficient to generate novel recombinant genes (or gene-clusters) that encode novel resistance specificities (unequal crossing-over and gene-conversion), there appears to be a more important mechanism that can be regarded as a process of adaptive evolution responsible for fine-tuning of novel recognition specificities. Purifying and diversifying selection, that is selection against and in favour of certain amino acid substitutions, is expressed as the ratio between non-synonymous (K_a) to synonymous (K_s) nucleotide substitutions. Examination of different types of tandemly repeated genes suggests that the LRR domain shows much higher

levels of diversity that can be explained by sequence exchange per se, particular at predicted solvent-exposed sites than other domains within the genes (Parniske *et al.*, 1997; Botella *et al*., 1998; Wang *et al*., 1998; Caicedo *et al*., 1999; Noël *et al*., 1999; Cooley *et al*., 2000; Van der Vossen *et al*., 2000). However, it is unknown how the hypervariability in the solvent exposed-LRR region is accomplished.

Durability of resistance genes

The ongoing battle between plants that develop novel resistance genes and pathogens that circumvent recognition by these plants is regarded as an arms race. It implies that *R* genes are relatively young. However, recent reports show *R* gene longevity and co-existence of multiple *R* genes conferring recognition of the same elicitor in natural plant populations (Caicedo *et al*., 1999; Stahl *et al*., 1999; Bergelson *et al*., 2001; Riely and Martin, 2001; Van der Hoorn *et al*., 2001a). This suggests that *R* genes are maintained by balancing selection, which occurs when loss of the matching *Avr* gene in the pathogen is associated with a reduced pathogenic fitness. Thus, an *R* gene becomes prevalent as a result of its selective advantage, whereas the frequency of such an *R* gene is reduced when the corresponding pathogen causes less disease pressure. This balancing selection implies the existence of two counteracting forces: a cost of virulence for the pathogen and a cost of resistance for the host.

The durability of disease resistance is affected by the evolutionary potential of the pathogen population as pathogens with a high evolutionary potential are more likely to overcome genetic resistance than pathogens with a low evolutionary potential (McDonald and Linde, 2002). Additionally, the virulence role of the Avr factor is crucial for balancing selection. Without any virulence function of the Avr factor, the selection pressure on the pathogen will result in negative selection on the *Avr* gene. As a result, the original *Avr* gene will become rare or even extinct. Although *Avr* genes have been identified as avirulence determinants, their primary function is expected to be associated with pathogenic or saprophytic fitness rather that with avirulence. Indeed, many Avr factors contribute to virulence of the pathogen (Van 't Slot and Knogge, 2001), but their relative contributions are often redundant and difficult to assess. Avr factors with a virulence role will be maintained in the pathogen population even though this will result in avirulence on a subpopulation of the host. This is for example demonstrated for *AvrXa7* of the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (Vera Cruz *et al*., 2000) and *avrBs2* of *Xanthomonas campestis* pv. *vesicatoria*.

THE *CLADOSPORIUM FULVUM* - TOMATO INTERACTION

Introduction

Cladosporium fulvum is a non-obligate biotrophic fungal pathogen, which causes leaf mold of tomato (*Lycopersicon* spp.) (Figure 2). This pathosystem serves as a model to study the molecular basis of gene-for-gene interactions between plants and pathogenic fungi. Conidia of *C. fulvum* germinate on the leaf surface and enter the leaves through stomata and the fungus obtains nutrients via thickened intercellular hyphae that are in close contact with the host mesophyll cells. No specialised feeding structures, such as haustoria, are formed during infection. About two weeks after penetration, when the intercellular spaces are fully colonised, conidiophores emerge from the stomata and numerous conidiospores are released that can cause secondary infections.

The *Avr* and *Ecp* genes of *C. fulvum*

During colonisation, *C. fulvum* secretes at least ten different small, cysteine-rich proteins into the apoplastic space. Eight of these are cloned (Joosten and De Wit, 1999; Laugé *et al.*, 2000; Luderer *et al.,* 2002a; Westerink *et al.*, 2002). The known Avr proteins (Avr2, Avr4, Avr5, Avr4E and Avr9) trigger hypersensitive response (HR)-associated defence responses in tomato plants with the matching *Cf* resistance genes (reviewed by Joosten and De Wit, 1999). For *Avr5* the corresponding cDNA has not been cloned yet. Numerous races of *C. fulvum* exist that are able to overcome one or more specific *Cf* resistance genes. This can be the result of deletions, point mutations or insertion of a transposon either resulting in complete absence of the encoded Avr protein or by the production of unstable forms of proteins that are rapidly degraded, and subsequently allowing the fungus to circumvent plant defence responses (Joosten and De Wit, 1999, Luderer *et al*., 2002a; Westerink *et al*., 2003). Often, loss of the Avr proteins appears not to result in a visible fitness penalty for the pathogen. As a result, resistance by *Cf* genes recognising Avr proteins which are not essential to the pathogen is not expected to be durable.

In contrast to the four race-specific Avr proteins, race-specificity has not yet been observed for the remaining proteins called extracellular proteins (Ecp1, Ecp2, Ecp3, Ecp4 and Ecp5). *Ecp3* has not been cloned yet. Each individual Ecp protein is produced by all known strains of *C. fulvum,* suggesting that these play a role in pathogenic fitness. Indeed, disruption of *Ecp1* and *Ecp*2 resulted in reduced virulence of a *C. fulvum* transformant demonstrating that at least Ecp1 and Ecp2, are virulence factors (Laugé *et al*., 1997).

Because of the importance for the pathogen, resistance based on recognition of virulence factors by the plant is expected to be more durable as loss of these factors will result in a fitness penalty for the pathogen. Individual accessions within the *Lycopersicon*

genus could be identified that react with an hypersensitive response (HR) to each of the individual Ecp proteins. Matching *R*-genes, designated *Cf-Ecps*, have been introgressed into cultivated tomato (*L. esculentum*) for further analysis (Laugé *et al.*, 1998a; Haanstra *et al.*, 2000; Laugé *et al.*, 2000).

Additionally, HR-associated recognition of Ecp2 was found in non-host plants of *C. fulvum.* Laugé *et al.* (2000) showed by using PVX-mediated expression of the elicitor genes that two of the three accessions of *Nicotiana paniculata* respond with an HR to Ecp2. These results indicate that these plants recognise Ecp2-like proteins and may indicate that these plants contain a functional *Cf-Ecp* gene.

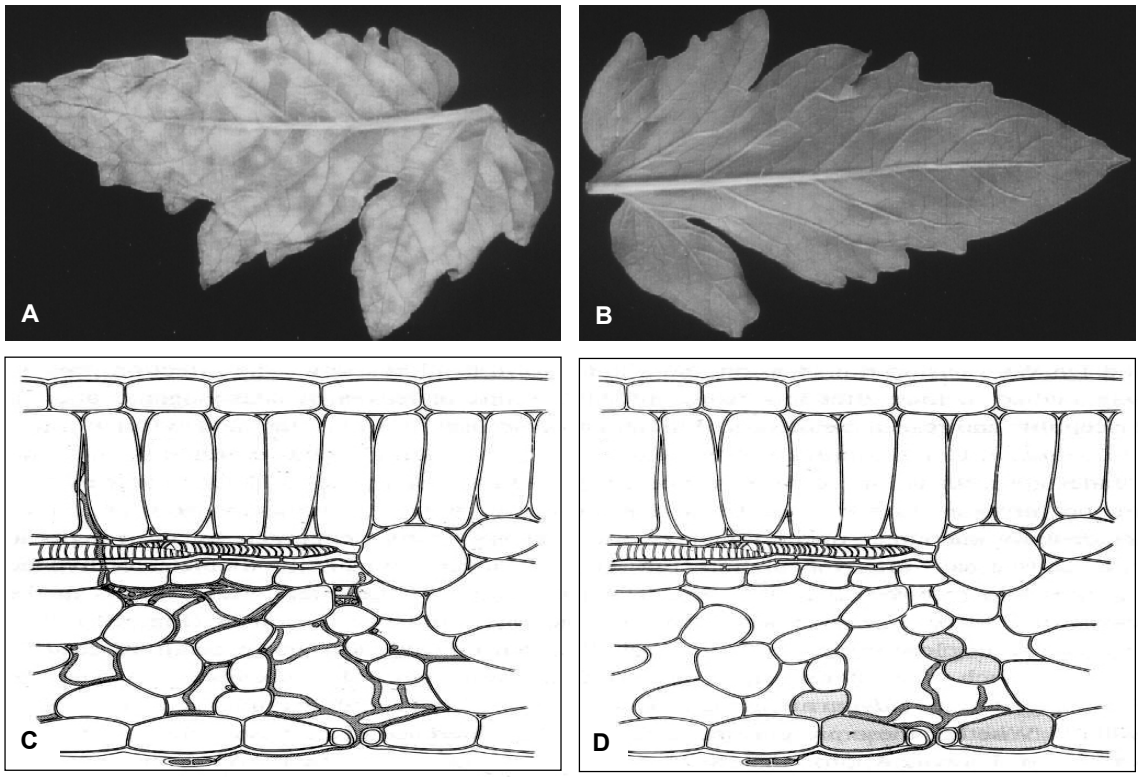

Figure 2. The compatible and incompatible interaction between tomato and *Cladosporium fulvum*. (**A**) Lower side of a leaf of a susceptible tomato plant, two weeks after inoculation with a virulent strain of *C. fulvum* (compatible interaction). (**B**) Lower side of a leaf of a resistant tomato plant, two weeks after inoculation with an avirulent strain of *C. fulvum* (incompatible interaction). (**C**) Schematic representation of a cross-section of a susceptible tomato leaf 6-8 days after inoculation with a virulent strain of *C. fulvum*. The fungal conidiospore germinates on the lower leaf surface, forms a thin runner hyphae and enters the leaf through a stoma. The penetration hyphae develops into a thicker intercellular mycelium and proliferates abundantly in the intercellular spaces around the mesophyl cells; no visible responses are observed. (**D**) Resistance cultivar inoculated with an avirulent race of *C. fulvum*. After penetration fungal growth is arrested and mesophyll cells in contact with the fungus develop a hypersensitive response (HR, dark cells).

The *Cf* genes of tomato

A number of *Cf* resistance genes have been mapped at four different loci. *Cf-2* and *Cf-5* are closely linked on Chromosome 6 (Dixon *et al.,* 1996, 1998), *Cf-4*, *Cf4E*, and *Cf-9* have been mapped on the short arm of Chromosome 1 at the *Milky Way* (*MW*) locus (Van der Beek *et al.,* 1992; Balint-Kurti *et al.,* 1994; Takken *et al.,* 1998), *Cf-Ecp2* and *Cf-Ecp3* at the *Orion* (*OR*) locus, twelve cM proximal to the *MW* locus (Haanstra *et al.,* 1999; Yuan *et al.,* 2002), and *Cf-Ecp5* at the *Aurora* locus, four centiMorgan (cM) proximal to the MW cluster (Haanstra *et al.,* 2000) (Figure 3). Several *Cf* genes, notably *Cf-9* (Jones *et al.,* 1994), *Cf-2* (Dixon *et al.,* 1996), *Cf-4* (Thomas *et al.,* 1997), *Cf-4E* (Takken *et al.,* 1998), *Cf-5* (Dixon *et al.,* 1998), and *Cf-9DC* (Van der Hoorn *et al.,* 2001a, M. Kruijt, *pers. comm.*) have been cloned and sequenced. The *Cf*-genes encode membrane-anchored, cytoplasmic glycoproteins of which the extracytoplasmic domain mainly consists of leucine-rich repeats (LRRs) which are predicted to mediate recognition of matching fungal elicitor proteins. The *Cf* genes cloned so far belong to two gene families, the *Cf* genes located in the *MW* locus (*Cf-4*, *Cf4E*, *Cf-9,* and *Cf-9DC*) are very homologous to each other and are referred to as *Hcr9*s (Homologues of the *C. fulvum* resistance gene *Cf-9*). The genes *Cf-2* and *Cf-5* are similarly referred to as *Hcr2*s. Depending on the genotype, the *MW* locus can contain up to six *Hcr9*s (Parniske *et al.,* 1997, 1999; Parniske and Jones, 1999; M. Kruijt, *pers. comm.*). The short arm of Chromosome 1 harbours two additional thoroughly characterised clusters with *Hcr9*s, *Northern Light* (*NL*) and *Southern Cross* (*SC*) (Parniske *et al.,* 1999), but to our current knowledge these clusters do not contain functional *Cf* genes. RFLP analysis with a *Cf-9* probe showed that the the *Cf-Ecp* genes in the *AU* and *OR* loci also comprise *Hcr9*s (Haanstra *et al.,* 1999*,* 2000; Yuan *et al.,* 2002). So far, 19 *Hcr9*s have been sequenced. Sequence variation within Cf proteins is generally present in the region encoding the first 16 LRRs that most probably determines recognition specificity (Thomas *et al.,* 1997; Van der Hoorn *et al.,* 2001b).

OUTLINE OF THESIS

The research described in this thesis focuses on the molecular characterisation of the recognition of extracellular proteins (Ecps) of *C. fulvum* mediated by *Cf-Ecp* genes in the host species tomato, and by a *Cf-Ecp*-like locus in *Nicotiana* non-host species. Additionally, the sequence variation present in elicitors is studied at *C. fulvum* population level. These results provide a scientific basis for the development of co-evolution and the durability of the corresponding *Cf-Ecp* genes.

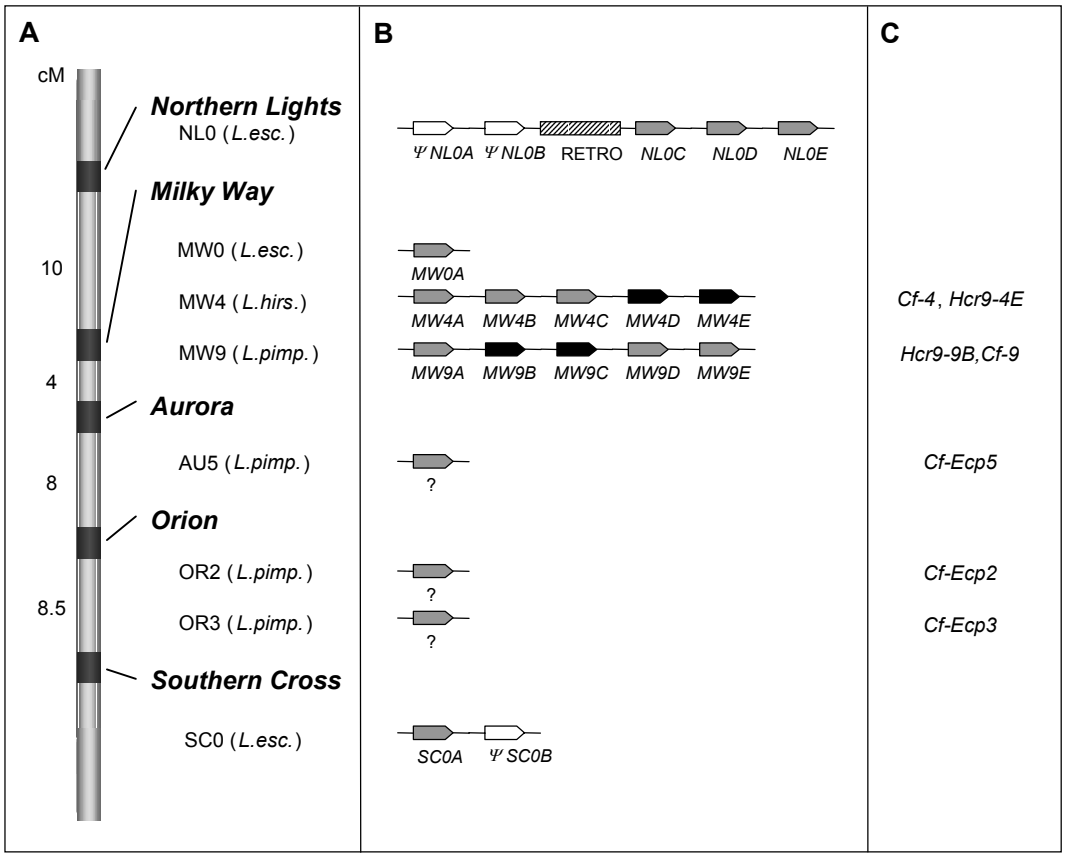

Figure 3. Map position, genetic distance (cM) and physical structure of the *Northern Lights* (*NL*), *Milky Way* (*MW*), *Aurora* (*AU*), *Orion* (*OR*) and *Southern Cross* (*SC*) loci harbouring clusters of *Hcr9*s on the short arm of Chromosome 1. (**A**) A genetic map of the various clusters showing the position of five *Hcr9* loci relative to each other. (**B**) The physical organisation of previously studied *Hcr9* clusters is shown. (**C**) The *Cf* resistance genes present in the cluster are indicated. Arrowed boxes indicate the relative position and orientation of *Hcr9*s; white arrowed boxes: *Hcr9* pseudogenes; grey arrowed boxes: *Hcr9*s with unknown function; black arrowed boxes: known *Hcr9* resistance genes. *Hcr9* clusters are derived from different haplotypes: NL0: *L. esculentum* Cf0; MW0: *L. esculentum* Cf0; MW4: *L. hirsutum* Cf4; MW9: *L. pimpinellifolium* Cf9;. The organisation of the *Orion* and *Aurora* cluster is unknown. RETRO denotes a retrotransposon insertion in the *NL* cluster.

The main part of this thesis focuses on the cloning of the *Cf-Ecp2 OR* cluster and the identification of the *Cf-Ecp2* resistance gene that mediates HR-mediated resistance upon Ecp2 recognition. The first part of the *Cf-Ecp2* trilogy, CHAPTER 2, describes the map- and homology-based cloning of the *OR Hcr9* cluster. We optimised a method to generate clone-specific fingerprint data which subsequently was used in the efficient calculation of contigs. Three *Hcr9*s were identified as candidate genes for *Cf-Ecp2*. By a PCR-based cloning approach, based on specific *OR* sequences, orthologous *Hcr9* genes were identified from different *Lycopersicon* species and haplotypes. Sequence homologies, protein characteristics and evolutionary relationships between the *Orion Hcr9s* are discussed.

To confirm whether the isolated *OR Hcr9* cluster was complete, in CHAPTER 3 we describe the development and the application of a resistance gene analogue (RGA) fingerprinting method. The RGA fingerprint method enabled us to generate labelled *Hcr9*-specific markers that could be analysed on a LI-COR DNA sequencer. With this method RGA-markers were identified that cosegregate with resistance. In addition, this method enabled us to determine whether individual *Hcr9* genes are expressed.

In the third part of the *Cf-Ecp2* trilogy, CHAPTER 4, we describe the different methods to identify the functional *Cf-Ecp2* gene. Induction of the HR upon Ecp2 recognition by candidate gene products was investigated by the transient expression of candidate genes in *Nicotiana* species and by complementation analysis in susceptible tomato. Based on the results, we hypothesise why Ecp2 recognition mediated by one of the three candidate *Cf-Ecp2* genes could not be observed.

Little is known of the genetic basis of non-host resistance and the perception of elicitor proteins. It is unknown whether non-host based HR is functionally related to resistance or whether it is a consequence of spontaneous evolution of *R*-genes in non-host plants which frequently results in recognition of non-self proteins. In CHAPTER 5 we focus on the Ecp2 recognition in non-host plants. HR-associated recognition of Ecp2 is present in several non-related *Nicotiana* species that are all non-host plants of *C. fulvum*. In addition, the effects of Ecp2-recognition on the growth of *C. fulvum* on *Nicotiana* plants were studied. Finally, the genetic and molecular basis of Ecp2-recognition in *Nicotiana* species was investigated.

The Ecotilling mutation detection method was used to compare the sequence variation in ribosomal internal transcribed spacers (ITS) with the variation in Avr and Ecp elicitor proteins in strains of *C. fulvum* that have been collected world-wide (CHAPTER 6). Results on the sequence variation, and the types of mutations in different elicitors were used to hypothesise about the biological relevance of the elicitors, the selection pressure on *C. fulvum* by host plants and durability of *Cf* resistance genes.

The thesis is concluded with a general discussion on Cf- and Cf-like proteins involved in disease resistance (CHAPTER 7). The current knowledge on the genetics and evolution of *Cf* genes, Cf protein characteristics, elicitor perception and signal transduction in the tomato - *C. fulvum* pathosystem is reviewed. In addition, the current knowledge on *Cf*-like proteins that are involved in other pathosystems is presented. Finally, this chapter suggests some future directions in research on the described pathosystems.

Chapter 2

The tomato *Orion* locus comprises an unique class of *Hcr9* genes

This chapter is submitted for publication to *Molecular Breeding*.
Co-auteurs: Bas F. Brandwagt, Guusje Bonnema, Pierre J.G.M. de Wit, and Pim Lindhout.

ABSTRACT

Resistance against the tomato fungal pathogen *Cladosporium fulvum* is often conferred by *Hcr9* genes (Homologues of the *C. fulvum* resistance gene *Cf-9*) that are located in the *Milky Way* cluster on the short arm of Chromosome 1. These *Hcr9* genes mediate recognition of fungal avirulence gene products. In contrast, the resistance gene *Cf-Ecp2* mediates recognition of the virulence factor Ecp2 and is located in the *Orion* (*OR*) cluster on the short arm of Chromosome 1. Here, we report the map- and homology-based cloning of the *OR Hcr9* cluster. A method was optimised to generate clone-specific fingerprint data that were subsequently used in the efficient calculation of genomic DNA contigs. Three *Hcr9*s were identified as candidate *Cf-Ecp2* genes. By PCR-based cloning using specific *OR* sequences, orthologous *Hcr9* genes were identified from different *Lycopersicon* species and haplotypes. The *OR Hcr9s* are very homologous. However, based on the relative low sequence homology to other *Hcr9*s, the *OR Hcr9*s are classified as a new subgroup. As a consequence, the origin and the mode of action of this unique class of *Hcr9*s may differ from the other *Hcr9*s.

INTRODUCTION

The fungal pathogen *Cladosporium fulvum* causes tomato leaf mould. In wild related species of tomato (*Lycopersicon esculentum*), many accessions have been identified that are resistant to *C. fulvum*. Plant breeders have introgressed the *C. fulvum* resistance genes from these accessions (designated *Cf*-genes) into new cultivars, which provide effective protection of tomato against the fungus. The tomato - *C. fulvum* interaction has been extensively used as a model to study gene-for-gene interactions (Joosten and De Wit, 1999). According to this model, resistance against the pathogen depends on the presence of at least two components: a resistance (*R*) gene in the plant and a matching avirulence (*Avr*) gene in the pathogen. An interaction between the resistance gene product and the Avr factor will invoke defence responses (accompanied a hypersensitive response; HR) eventually leading to complete inhibition of fungal growth.

Colonisation of tomato leaves by *C. fulvum* remains restricted to the apoplast and, consequently, the exchange of molecular signals between fungus and the plant occurs extracellularly. Detailed analyses of apoplastic fluids resulted in the identification, molecular isolation and characterisation of race specific Avr factors (reviewed by Joosten and De Wit, 1999; Luderer *et al.,* 2002a; Westerink *et al.,* 2003). In addition to the race-specific Avr factors, many other fungal low molecular weight peptides have been purified from apoplastic fluids from infected tomato leaves. Several of these corresponding Ecps

(Extracellular Proteins, including Ecp1, Ecp2, Ecp3, Ecp4 and Ecp5) have been isolated and, except for Ecp3, the encoding genes have been isolated (Van den Ackerveken *et al.,* 1993; Laugé *et al.,* 2000). During pathogenesis, all strains of *C. fulvum* produce and secrete these Ecps abundantly. Inoculation of an Ecp2-deficient replacement mutant on susceptible tomato plants showed a reduced virulence and induced accumulation of pathogenesis-related (PR) proteins (Laugé *et al.,* 1997). Consequently, Ecp2 was proposed to play a role in virulence of *C. fulvum* by suppression of host defence responses.

Various breeding lines and accessions of *L. pimpinellifolium* have been identified that recognise Ecps after injection of these proteins or by Potato Virus X-based expression of the corresponding cDNA in infected plant tissue (Laugé *et al.,* 1998a, 2000). The ability to recognise Ecp2 and to induce an HR is based on the single dominant gene *Cf-Ecp2* that confers resistance to *C. fulvum* strains producing the Ecp2 protein (Haanstra *et al*., 1999). As the *Cf-Ecp2* gene mediates recognition of the potential virulence factor Ecp2, it was hypothesised that *Cf-Ecp2* may confer durable resistance against *C. fulvum* (Laugé *et al*., 1998a). Furthermore, Ecp2 is also specifically recognised by several *Nicotiana* species which are non-hosts to *C. fulvum* (Laugé *et al.,* 2000; De Kock *et al.,* CHAPTER 5). This indicates that recognition of Ecp2 is widespread and can be found in both host and non-host plants.

A number of *Cf* resistance genes have been mapped at four different loci. *Cf-2* and *Cf-5* are closely linked and map on Chromosome 6 (Dixon *et al.,* 1996, 1998), *Cf-4*, *Cf4E*, and *Cf-9* have been mapped on the short arm of Chromosome 1 at the *Milky Way* (*MW*) locus (Van der Beek *et al.,* 1992; Balint-Kurti *et al.,* 1994; Takken *et al.,* 1998), *Cf-Ecp5* at the *Aurora* (*AU*) locus, four cM proximal to the *MW* cluster (Haanstra *et al.,* 2000), and *Cf-Ecp2* and *Cf-Ecp3* at the *Orion* (*OR*) locus, twelve centiMorgan (cM) proximal to the *MW* locus (Haanstra *et al.,* 1999; Yuan *et al.,* 2002), see also Figure 5. Several *Cf* genes, notably *Cf-9* (Jones *et al.,* 1994), *Cf-2* (Dixon *et al.,* 1996)*, Cf-4* (Thomas *et al.,* 1997), *Cf-4E* (Takken *et al.,* 1998), *Cf-5* (Dixon *et al.,* 1998), and *9DC* (Van der Hoorn *et al.,* 2001a; M. Kruijt, *pers. comm.*) have been cloned and sequenced. The *Cf*-genes encode membrane-anchored, cytoplasmic glycoproteins with an extracellular domain mainly consisting of leucine-rich repeats (LRRs) which are predicted to mediate recognition of matching fungal elicitor proteins. The *Cf* genes cloned so far belong to two gene families, the *Cf* genes located in the *MW* locus (*Cf-4*, *Cf4E*, *Cf-9,* and *9DC*) are very homologous and are referred to as *Hcr9*s (Homologues of the *C. fulvum* resistance gene *Cf-9*) and similarly, the genes *Cf-2* and *Cf-5* are referred to as *Hcr*2s. Depending on the genotype, the *MW* locus can contain up to six *Hcr9*s (Parniske *et al.,* 1997, 1999; Parniske and Jones, 1999; M. Kruijt, *pers. comm.*). The short arm of Chromosome 1 harbours two additional clusters with *Hcr9*s, *Northern Lights* (*NL*) and *Southern Cross* (*SC*) (Parniske *et*

al., 1999). The latter clusters do not contain *Cf* genes involved in resistance. RFLP analysis with a *Cf-9* probe demonstrated that *AU* and *OR* loci containing the *Cf-Ecp* genes also comprise *Hcr9*s (Haanstra *et al.,* 1999*,* 2000; Yuan *et al.,* 2002). So far, 19 *Hcr9*s have been sequenced. Sequence variation within Cf proteins is generally present in the first 16 LRRs that most probably determines recognitional specificity (Thomas *et al.,* 1997; Van der Hoorn *et al.,* 2001b).

The resistance gene *Cf-Ecp*2 has been accurately mapped on Chromosome 1 at < 0.3cM distance from the Cleaved Amplified Polymorphic Sequence (CAPS) marker CT116 (Haanstra *et al.,* 1999). Bonnema *et al.* (1997) showed by using a cross between *L. esculentum* and *L. peruvianum* LA2157, that near the CT116 locus a genetic distance of 1 cM corresponds to a physical distance of maximally 52 kb. The tight linkage of *Cf-Ecp2* with CT116 and the cosegregation with *Hcr9*s prompted us to use a combination of a homology-based- and map-based cloning approach for *Cf-Ecp2*.

In the present study, the cloning and sequence analysis of the *Hcr9*s at the *OR* cluster is reported. Additionally, the presence of orthologous *OR* clusters was investigated. We isolated *OR-Hcr9*s from other tomato haplotypes and discuss unique features of these *Hcr9*s and corresponding clusters.

RESULTS

Library construction and screening

The *Cf-Ecp2* gene has previously been mapped within approximately 20 kb from the genetic marker CT116 (Haanstra *et al*., 1999). A five genome equivalent library was constructed in the pCLD04541 binary cosmid vector (Bent *et al.,* 1994) with an average insert size of approximately 20 kb. Isolation of library clones containing *Hcr9*s and/or CT116 would enable us to isolate an overlapping series of clones (contig) covering the *OR* resistance cluster.

Due to a high background signal, hybridisation screening of pooled cosmid clones with *Cf-* and CT116 probes was not successful. Therefore, a PCR-based screening using *Hcr9-* and CT116 specific primer sets was performed. In total, 23 cosmids harbouring *Hcr9* sequences and one cosmid containing the CT116 marker were identified. Detailed characterisation of these cosmids by restriction mapping, DNA hybridisation and sequence analysis of *Hcr9*-derived PCR amplification products indicated that coverage of clones varied over the genome (data not shown). Many cosmids showed similar sequences to known *Hcr9* genes located in *NL*, *MW* or *SC* cluster. Two cosmids were identified that contained *Hcr9*s of unknown origin. Unfortunately, physical overlap of these clones with the clone containing CT116 was not present.

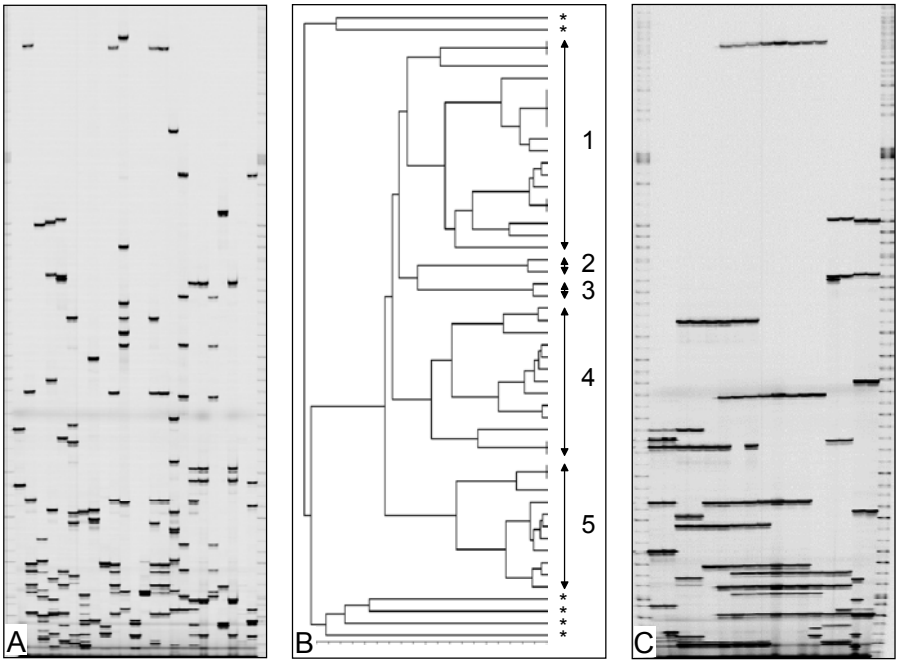

Figure 1. Ordering of library clones in contigs by PCR-fingerprinting and distance trees. (**A**) PCR fingerprint pattern of randomly ordered *Eco*RI/*Mse*I-mediated pBlueStar library clones containing *Hcr9*s and/or CT116 (subset of clones). The fingerprint pattern of each clone was converted to a binary data set (presence or absence of a band), which, together with additional experimental data (not shown), enabled the calculation of a distance tree using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) (**B**). Distance tree in which overlapping library clones were clustered. Arrows indicate five clades with contiguous clones. Clade 1 represents the *Northern Lights* cluster; clade 2 the *Milky Way* cluster, clade 3 is a cluster of unknown origin; clade 4 the *Orion* cluster; clade 5 the *Southern Cross* cluster. Clones, which could not be assigned to a contig, are indicated with an asterisk; (**C**) Fingerprint pattern of contiguous PCR fingerprinted library clones.

We used the binary cosmid vector since it allows a direct *Agrobacterium*-mediated transfer of cloned plant DNA into plant cells for complementation experiments. Although a contig covering the complete *OR* locus could not be constructed, we continued with the functional analysis. Cosmids carrying candidate *Cf-Ecp2* genes were transformed into *Agrobacterium tumefaciens* strain GV3101. Restriction analysis of several transformed cosmids (including 3.8G and 4.8G, located in the *OR* contig) showed deletions of insert-DNA (results not shown). This artefact is probably caused by the recombination of duplicated homologous sequences on the insert and hampered a straightforward functional analysis of candidate *Cf-Ecp2* genes.

A second genomic library was made in the λ-BlueStar vector (Novagen) which enables the screening of phages by DNA hybridisation and subsequent conversion of the isolated phages into high-copy plasmids (pBlueStar). A 16-genome equivalent library was constructed aiming to cover the complete *OR* cluster despite even in case of biased genome coverage. Hybridisation screening of this library resulted in 49 individual *Hcr9* clones and six CT116 clones, of which four clones contained both the CT116 CAPS

marker and an *Hcr9* sequence confirming the tight linkage of CT116 with at least one *Hcr9*.

Contig construction and mapping

We expected that isolated library clones would align in different contigs corresponding to the different *Hcr9* clusters. Contig construction of the clones by (low-resolution) restriction mapping was very laborious and therefore a restriction-mediated PCR fingerprinting method was developed to obtain clone-specific, high-resolution fingerprints that facilitated a reliable contig establishment. Indeed, contiguous clones with similar fingerprint-patterns were clustered into a distance tree comprising distinct branches (Figure 1).

This restriction-mediated PCR fingerprinting method was initially optimised for the *Eco*RI/*Mse*I restriction enzyme combination. Additional fingerprint data were obtained by using the five/four-cutter restriction enzyme combination *Apo*I/*Mse*I which allowed the unambiguous location of clones into one contig. Some of the clones appeared in separate clades with unrelated fingerprints and could not be assigned to *Hcr9* contigs. Additional hybridisation and PCR analysis showed that these clones did not contain *Hcr9* sequences and were false-positives from the library screening. Positioning fingerprinted clones according to the position in this tree enabled us to visually confirm the order of clones in a contig (Figure 1). With this method we could efficiently construct five contigs.

Positioning of contigs on the tomato genetic map

Based on the sequence of *Hcr9*-derived PCR products, three contigs were assigned to the *NL*, *MW*, or *SC Hcr9* cluster. The fourth contig, spanning a 41 kb genomic DNA region, comprised the CT116 marker and therefore covers the *OR* locus (Figure 2a). The fifth contig with two overlapping clones contained one *Hcr9* of unknown origin. Physical overlap with one of the other contigs was absent. This contig may represent the *AU* locus of the *Cf-Ecp2* haplotype.

The *Cf-Ecp2 Orion* locus

The *Cf-Ecp2 OR* cluster represented by pBlueStar clones 19, 49 and J was completely sequenced. In the initial physical alignment, clone 19 was overlapping with clone 49 by three shared *Apo*I/*Mse*I markers. Remarkably, the consensus DNA sequence of clone 19 and 49 could not be aligned. Analysis of both sequences showed that this was due to a perfect DNA repeat encompassing both clones. Therefore, additional selected cosmid clones were fingerprinted to assign them to *Hcr9* contigs. After additional restriction mapping, DNA hybridisation, and sequencing of *Hcr9*-derived PCR products, three binary cosmid clones were identified which fitted in the *OR* contig. As a result, clone 19 and 49 showed to be separated by a 1 kb gap. A part of clone 4.8G was PCR-amplified and

sequenced to close this sequence gap. Finally, a complete 29 kb sequence was obtained for the complete *Cf-Ecp2 OR* cluster.

Four putative open reading frames (ORFs) present in the *Cf-Ecp2 OR* cluster are shown in Figure 2b. The CT116 CAPS marker is located in the first intron of a gene encoding the 26S proteasome regulatory subunit S12 (E-value 5e-21) and does not coffer the open reading frame. The 26S proteasome is a 2MDa proteolytic complex that degrades ubiquitinylated protein conjugates (Voges *et al*., 1999; Smalle *et al*., 2004). The proteasome pathway degrades proteins that arise from synthetic errors, spontaneous denaturation, free-radical-induced damage, improper processing or diseases (Hershko and Ciechanover, 1998). There is no evidence that this gene, of which the open-reading frame is very conserved in the plant kingdom, is involved in the recognition of the *C. fulvum* Ecp2 elicitor.

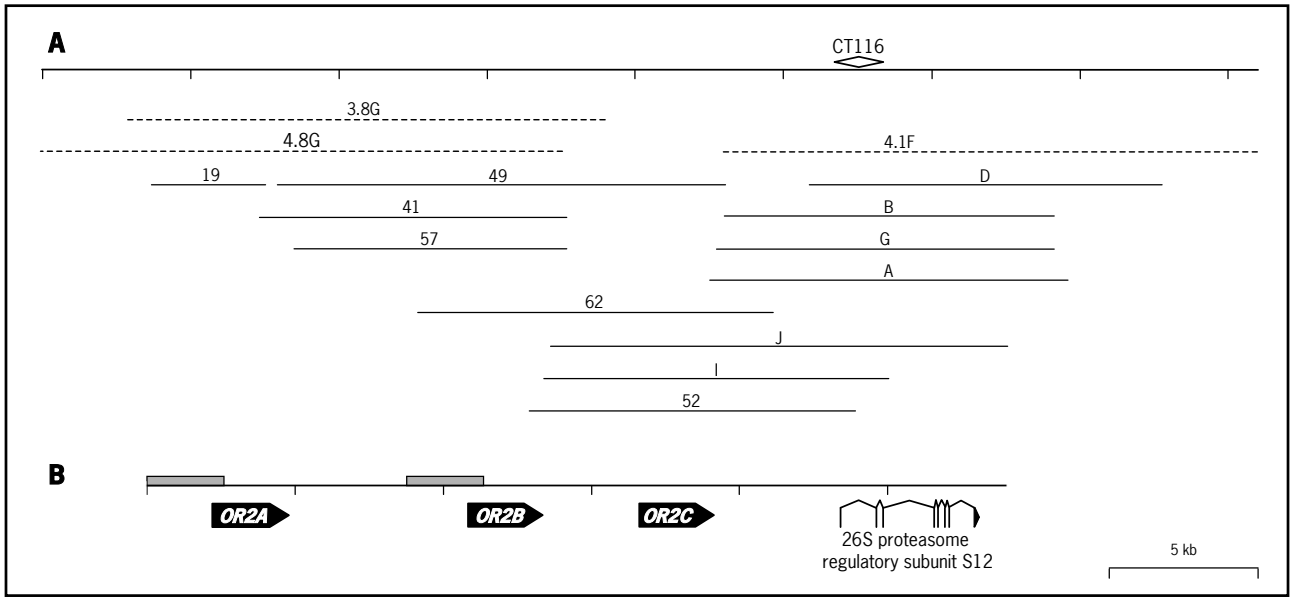

Figure 2. Physical map of the *Cf-Ecp2* locus. (**A**) A 41 kb contig spanning the *Orion Cf-Ecp2* resistance cluster was constructed with pBlueStar clones (solid lines) and pCLD04541 cosmid clones (dashed lines). The location of the CT116 CAPS marker is indicated by a diamond (◊). (**B**) A 30 kb segment was sequenced. The position and orientation of three intronless *Hcr9* ORFs is indicated by solid arrowed boxes. The position of the 26S proteasome regulatory subunit S12 exons are shown by connected boxes and triangle. The perfect tandem repeats are indicated by horizontal grey boxes.

Figure 3. Alignment of the Cf-9, OR2A, OR2B and OR2C proteins. Amino acid residues identical to Cf-9 are indicated with solid black background. Sequence gaps inserted to maintain the alignment are indicated by dashes. Domains are indicated above the sequence as follows: SP, signal peptide (A-domain); B, cysteine-rich domain, cysteine residues are indicated with an asterisk; LRR 1 to 27 (C-domain), various β-sheets are indicated (consensus xxLxLxx) each of which contains five solvent-exposed amino acid residues (x); D, domain without obvious features; E, acidic domain; TM, putative transmembrane domain; G, basic domain, representing the short, putative, cytoplasmic tail.


SP
B
LRR1
LRR2
LRR3
LRR4
LRR5
LRR6
LRR7
LRR8
LRR9
LRR10
LRR11
LRR12
LRR13
LRR14
LRR15
LRR16
LRR17
LRR18
LRR19
LRR20
LRR21
LRR22
LRR23
Loop Out
LRR 24
LRR25
LRR26
LRR27
D
E
TM
G
Cf-9
OR2A
OR2B
OR2C
ORE2A
ORE2B
ORE2C

Figure 3.

The major part of the *OR* sequence contains three genes homologous to *Hcr9*s. These encode membrane-anchored receptor-like proteins with 27 extracellular leucine-rich repeats (LRRs) and a short cytoplasmic tail (Figure 3). Following the nomenclature used by Parniske and Jones (1999), these genes were designated *OR2A*, *OR2B*, and *OR2C*, after their genetic location *Orion*, their putative involvement in Ecp2 recognition and the alphabetic order of the gene in the *OR* cluster. The *OR* cluster contains a 2621 bp perfect tandem repeat including the promoter region and the first part of the coding region (432 bp) of *OR2A* and *OR2B*.

Typical for Hcr9 proteins, the majority of the amino acid variation between members of this family is present in the B-domain and the first 17 LRRs of the C-domain. The alignment of the three newly identified Hcr9s shows that the amino-acid variation is spread throughout the protein (Figure 3). The signal sequence has only ± 50% amino-acid homology with Cf-9 but still resembles a putative signal peptide for extracellular targeting. The cysteine-rich B-domain of OR2A/OR2B has low sequence homology with Cf-9 but the cysteine residues are conserved. In OR2C, a stretch of 17 amino acids is absent, including one cysteine residue. In the majority of the first 17 LRRs, amino-acid substitutions occur at the putative solvent-exposed domains (xxLxLxx) of LRRs. The amino acid variation in the OR2 proteins continues in the C-terminal part, including LRRs 18-24, the loop-out, and the acidic E-domain.

Orthologous *OR* loci

To study the complexity of *OR Hcr9* clusters, an *Hcr9*-specific fingerprint method was developed (De Kock *et al*., CHAPTER 3). In a mapping population segregating for *Cf-Ecp2* resistance, certain *Hcr9* markers cosegregated with *Cf-Ecp2* resistance, while one *Hcr9* marker was in repulsion phase with *Cf-Ecp2* resistance. Probably, this *Hcr9* marker corresponds to an orthologous gene in the *OR* locus of the susceptible parent MM-Cf0.

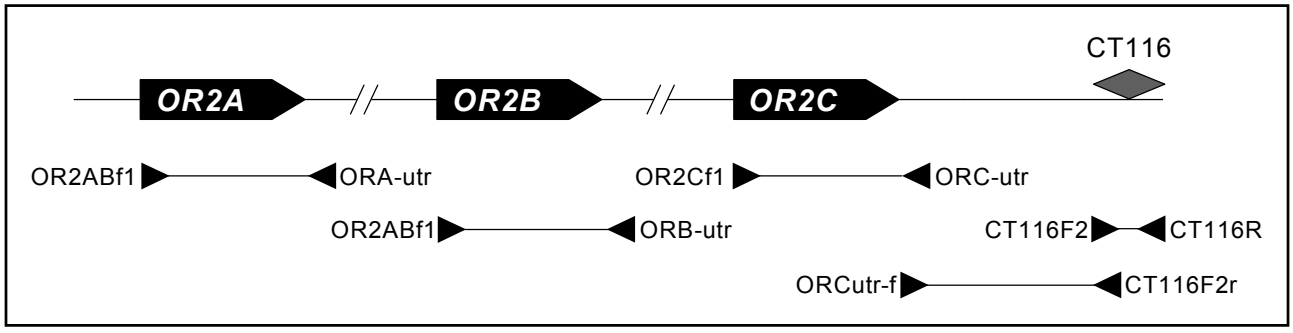

Figure 4. PCR-amplification strategy for the identification of *Orion* (*OR*) orthologous *Hcr9* genes and for the confirmation of physical linkage of *OR2C* orthologous genes with CT116. In the schematic representation of the *Cf-Ecp2 OR* cluster, the position and the orientation of the three *Hcr9*s are indicated by arrowed boxes, the CT116 CAPS marker is indicated by a diamond (◊). Triangles indicate annealing position and direction of primers. For further details see Experimental Procedures.

The *OR* clusters in other haplotypes were subsequently investigated. By a PCR-based cloning strategy using primer sets specific for each of the three *OR2s* (Figure 4), orthologous *OR Hcr9*s from Cf0-, Cf-Ecp3, and Cf-Ecp5 haplotypes were identified. Following the above nomenclature, these genes were designated after their genetic location *Orion*, the Cf-haplotype (Cf0*,* Cf-Ecp3, and Cf-Ecp5) and the alphabetic order of

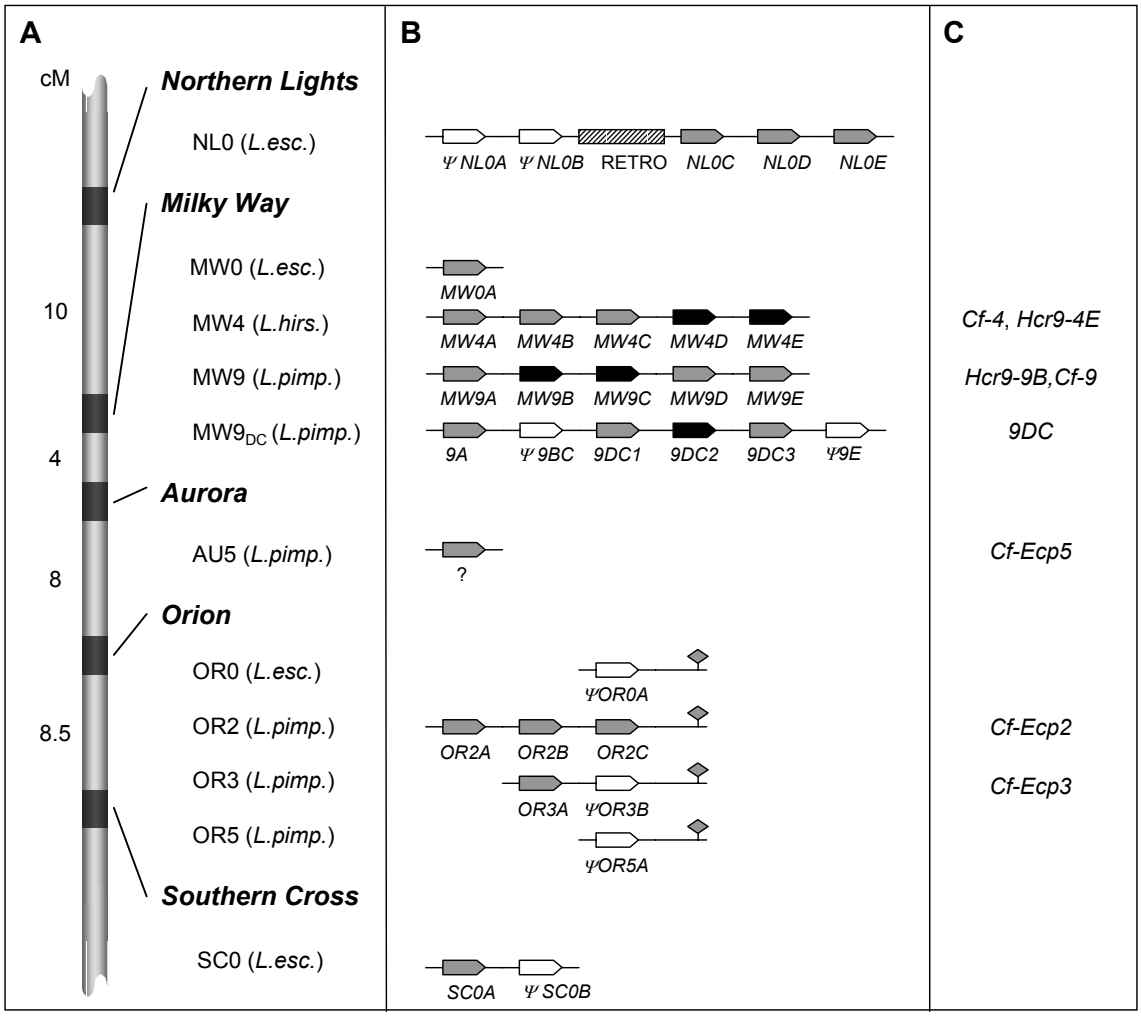

Figure 5. Map position, genetic distance (cM) and physical structure of the *Northern Lights*, *Milky Way*, *Aurora*, *Orion* and *Southern Cross* loci harbouring clusters of *Hcr9*s on the short arm of Chromosome 1. (**A**) A genetic map of the various clusters showing the position of five *Hcr9* loci relative to each other. (**B**) The physical organisation of each *Hcr9* cluster is shown. (**C**) 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: *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 (M. Kruijt, unpublished data). The organisation of the *OR* cluster was determined in this study: 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. The CT116 CAPS marker at the Orion locus is indicated by a diamond (◊); RETRO denotes a retrotransposon insertion in the *NL* cluster.

the gene in the *OR* cluster. The prefix Ψ indicates a pseudogene. The organisation of all the 32 presently known *Hcr9*s is depicted in Figure 5.

The *OR* orthologue in Cf-0, designated Ψ*OR0A*, is homologous to *OR2C* (99.7% sequence homology) but has a 10 bp deletion resulting in a frame shift. The MM-Cf-Ecp3 and MM-Cf-Ecp5 orthologues of *OR2C* (designated Ψ*OR3B* and Ψ*OR5A*, respectively) showed 99.5% and 99.1% sequence homology to *OR2C*. Both genes encode a truncated *Hcr9* caused by a point mutation resulting in a stop-codon (Cf-Ecp3 haplotype) or a nucleotide insertion (Cf-Ecp5 haplotype). By using primer sets specific for *OR2A*, no orthologous genes in other haplotypes were identified, while one orthologous gene in the *Cf-Ecp3 OR* cluster was identified by using a primer set specific for *OR2B* designated *OR3A*. The encoded protein showed 92% sequence homology to *OR2B.*

Phy sical linkage of *OR3A* and Ψ*OR3B* to the CT116 marker was proven by the analysis of *Cf-Ecp3* genomic library clones harbouring the *Cf-Ecp3 OR* locus (Y. Yinan, *pers. comm*.). Finally, physical linkage of Ψ*OR0A*, Ψ*OR3B*, and Ψ*OR5A* to CT116 was investigated by PCR analyses. The 4.2 kb DNA fragment that spans the distance between these *OR2C* orthologous genes and the CT116 locus could be PCR-amplified from all tested haplotypes. With these results we show the existence of orthologous gene clusters at the *OR* locus as was previously described for the *MW* cluster.

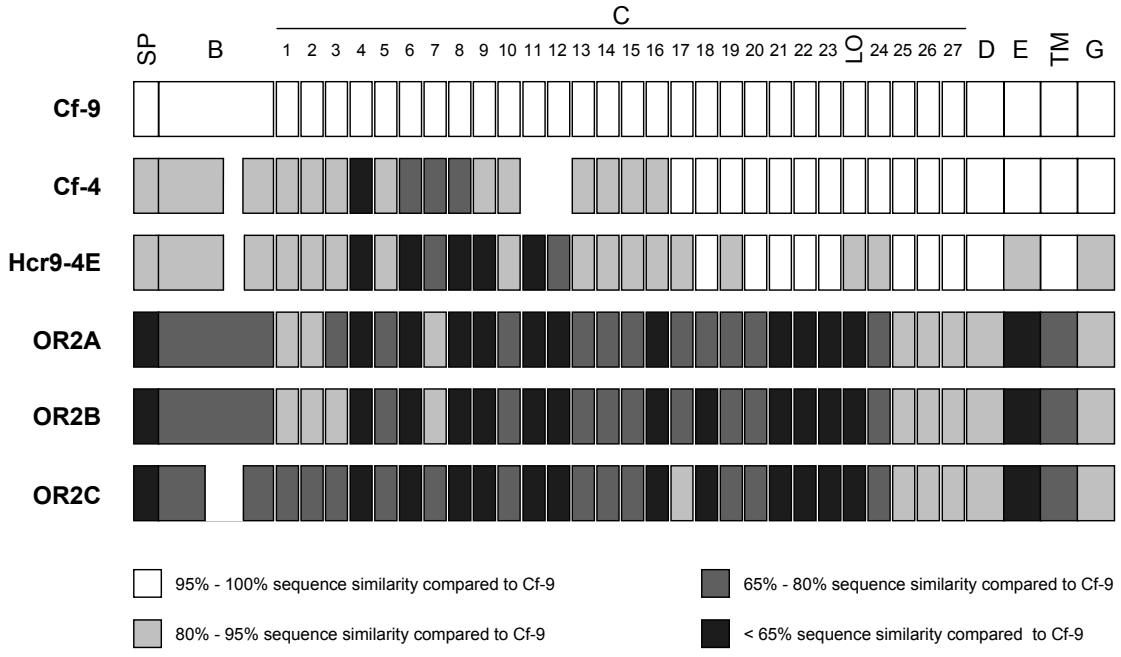

Figure 6. Schematic presentation of amino acid similarity of the *Cf* resistance proteins Cf-4, Hcr9-4E and the Orion Hcr9 protein OR2A, OR2B, and OR2C compared to the Cf-9 resistance protein; structural protein domains: SP, signal peptide; B, cystein-rich domain; C, domain containing 27 leucine-rich-repeats (LRRs), LO: Loop Out; D, domain without conspicuous features; E, acidic domain; TM, putative transmembrane domain; G, basic cytoplasmic domain. Grey scale indicates the level of amino acid similarity compared to the Cf-9 protein.

DISCUSSION

Isolation and characterisation of binary cosmid library clones

In a genomic library of more than five genome equivalents, statistically more than 99% of the genome should be covered by cosmid library clones. In our study, the binary cosmid library was incomplete at the *OR* locus. Therefore, it was not possible to isolate contiguous binary cosmid clones covering the complete *OR* cluster. Two additional reasons that prompted us to decide to construct a new genomic library in another vector were (*i*) the risk that *Cf-Ecp2* candidates that do not perfectly match to the degenerate *Hcr9* primers could have been missed in the PCR-selection of clones; (*ii*) DNA-instability of the cosmid inserts in *A. tumefaciens* hampered functional analysis of candidate *Cf-Ecp2* genes. Although the binary cosmid vector pCLD04541 has frequently been used for the cloning of resistance genes, e.g. *Cf-2*, *Cf-4*, *Cf-5*, *Hero* (Dixon *et al.,* 1996; Thomas *et al.,* 1997; Dixon *et al.,* 1998, and Ernst *et al.,* 2002), this vector was not suitable to clone *Cf-Ecp2*. To our knowledge, this observation has not been reported in literature before.

Isolation and contig construction of pBlueStar clones

To avoid the problems described above, a larger, 16-genome equivalent library in λBlueStar was made and phages were screened by DNA hybridisation. After restriction-mediated PCR fingerprinting and contig calculation, selected clones were positioned into five different *Hcr9*-containing contigs representing the known *NL*, *MW* and *SC Hcr9* clusters and the *OR Hcr9* cluster. It is possible that the fifth contig belongs to the *AU* locus. The restriction-mediated PCR fingerprinting and subsequent calculation of contigs appeared to be a very efficient and reliable method for contig construction. Our method follows the same strategy of FPC (fingerprinted contigs) described by Soderlund, *et al*. (1997), but a different type of fingerprint data is used and contig calculation is less complex. Selection of frequent or rare cutting restriction enzymes for fingerprinting is based on the required resolution and sizes of template DNA. After all, integration of two genomic libraries representing in total 21 genome equivalents was necessary for the contig construction and to sequence the *OR Hcr9* cluster.

The *Cf-Ecp2 Orion* cluster

The contig is at one side flanked by the CT116 CAPS marker, but the physical end on the other site of the contig remains obscure and thereby the number of *Hcr9*s was not known. To confirm the number of *Hcr9*s in the *OR* cluster, an *Hcr9* resistance gene analogue (RGA) fingerprint method was developed (De Kock *et al*., CHAPTER 3). With this method, all *Hcr9* markers which cosegregated with *Cf-Ecp2* resistance corresponded with the three

*Hcr9*s of the *OR* contig. Therefore, we concluded that the *Cf-Ecp2 OR* cluster contains three *Hcr9*s.

The *Cf-Ecp2 OR* cluster harbours a large duplication of 2.6 kb, which is probably a result of a recent intergenic unequal crossing over. Interestingly, the first 576 bp of this duplicated region shows high sequence homology (92%) with the promoter region of *Hero*, an NBS-LRR resistance gene located on Chromosome 4 conferring broad spectrum resistance against potato cyst nematodes (Ernst *et al.,* 2002). The 576 bp region is located in the promoter and may therefore act as cis-acting binding domain that regulates the transcriptional activity of the upstream gene.

The *OR* Hcr9 proteins show the characteristic domains of membrane-anchored, cytoplasmic glycoproteins of which the extracytoplasmic domain mainly consists of leucine-rich repeats (LRRs). The LRR domains of R proteins were suggested to be involved in the recognition of the corresponding elicitor or co-acting proteins (Jones and Jones, 1997). Consistent with this theory, it was found that Hcr9 proteins with specificity for different Avr factors predominantly differ in amino acid residues located at putative solvent-exposed positions in the N-terminal LRRs (Parniske *et al*., 1997, Van der Hoorn *et al.,* 2001b; Wulff *et al.,* 2001). However, in contrast to the previously known Hcr9 proteins, the variation in the *OR* Hcr9 proteins continues in the C-terminal LRRs. Additionally, the loop-out and the acidic E-domain vary, whereas these domains are rather conserved in the *NL*, *MW*, *SC* Hcr9 proteins (Figure 6). The loop-out of the BRI1 receptor protein, a receptor-like kinase located on the cell surface that is involved in brassinosteroid signalling (Li and Chory, 1997; Wang and He, 2004), was proven to facilitate brassinosteroid binding. Therefore, the abundant variation in the loop-out domain and the other C-terminal domains of the *OR* Hcr9s may indicate that elicitor perception and signal transduction mediated by the *OR* Hcr9s is different compared to the other Hcr9s.

Orthologous *Orion Hcr9* clusters

The low sequence homology at primer-annealing site of the *OR Hcr9*s compared to the consensus sequence of *Hcr9*s enabled us to design *OR* specific primers that were used to identify orthologous *OR* genes in other *Lycopersicon* accessions. The Cf-Ecp3 haplotype contains two *Hcr9*s (*OR3A* and Ψ*OR3B*) and the Cf-0 and Cf-Ecp5 *OR* loci both contain only one *Hcr9* (Ψ*OR0A* and Ψ*OR5A*, respectively). The Cf-Ecp3 and Cf-Ecp5 haplotypes were not studied by RGA-fingerprint analysis. Therefore, the presence of additional *Hcr9*s at these loci cannot be excluded. The orthologous genes of *OR2C* (which are Ψ*OR0A*, Ψ*OR3B* and Ψ*OR5A*) show a high sequence identity. *OR0A* is present in the Cf-0 *L. esculentum* haplotype, whereas the other genes are from different *L. pimpinellifolium*

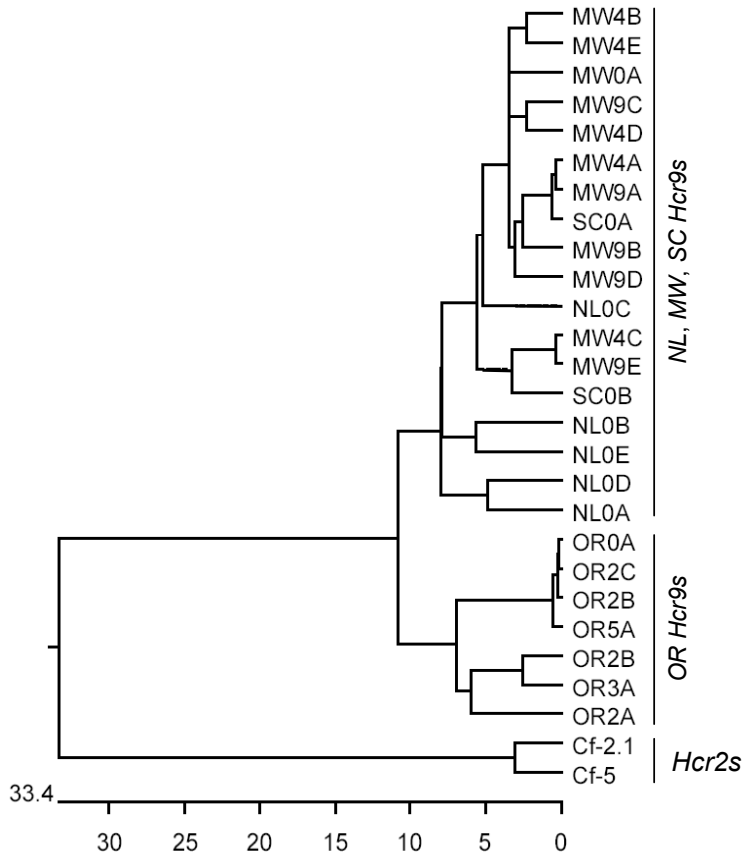

Figure 7. Phylogenetic relationships between *Hcr9*s and *Hcr2*s. The nucleotide sequences were aligned using the Clustal method (Higgins and Sharp, 1989) and the neighbour-joining method was employed to construct a phylogenetic tree. Three different clades are constructed representing (*i*) the *NL*, *MW*, *SC Hcr9*s, (*ii*) the *OR Hcr9*s, and (*iii*) the *Hcr2*s. The scale bar indicates the degree of nucleotide dissimilarity.

introgressions. Apparently, these sequences are very conserved in two *Lycopersicon* species although only *OR2C* encodes a full-length protein.

Polymorphic sites can distinguish the individual members and allow speculation about the origin and relation to other members of this gene family. The *OR Hcr9*s are highly homologous. Based on the shared polymorphic sites, the *OR Hcr9*s are subsequently most related to *ΨNL0A*, *ΨNL0B, NL0D*, and *NL0E*. The *Hcr9s* in the *OR* cluster represent a distinct subgroup of *Hcr9*s when the sequences are aligned with *NL*, *MW*, *SC Hcr9s* and *Hcr2*s (Figure 7). In contrast, the intergenic regions are very unique for the *OR* locus. The *MW* and *SC* clusters harbour several *LipoxygenaseC* (*LoxC*) exons that are thought to have coduplicated with *Hcr9*s (Parniske *et al*., 1997). These *LoxC* sequences are absent in the *OR* cluster as they are also absent in the *NL* cluster. Parniske and Jones (1999) suggested that the divergence of the *NL Hcr9*s was probably a consequence of its genetic isolation. This suggestion is now contradicted by our finding of the relative high homology of the *OR Hcr9*s to the *NL Hcr9s*, although the *OR* cluster is located between the *MW* and *SC* cluster.

Homology searches in databases using *Cf* gene and protein sequences showed that numerous sequences highly homologous to *Cf* genes (E value < 1e-50 at nucleotide level) are present in *Lycopersicon, Solanum*, and *Capsicum* species. Interestingly, sequences most homologues to the *OR Hcr9*s are not found in *Lycopersicon* species, but

are present in *Solanum tuberosum.* This suggests the existence of common ancestral *OR* genes before *Lycopersicon* and *Solanum* speciation and indicates that the unique features of the *OR Hcr9* genes remained conserved during evolution.

Depending on the haplotype, the identified *OR Hcr9*s are candidate genes for *Cf-Ecp2* and *Cf-Ecp3* function. Complementation analysis with these candidate *Cf-Ecp2* and *Cf-Ecp3* genes has to reveal which genes are involved in the perception of the *C. fulvum* elicitor Ecp2 and Ecp3, respectively, and trigger HR-based resistance.

EXPERIMENTAL PROCEDURES

pCLD04541 binary cosmid library construction and screening

Genomic DNA was isolated according to Van der Beek *et al.* (1992) from four-week-old leaves of the breeding line Ontario 7518 (Cf18) showing *Cf-Ecp2*-mediated resistance (Laugé *et al.,* 1998a). DNA was partially digested with *Sau*3A I to an average size of 40 kb. Partially filled-in insert DNA (1 µg) was ligated at 4°C for 16 hr in a total volume of 10 µl with 500 ng of *Xho*I digested and partially filled-in binary cosmid vector pCLD04541 (Dixon *et al*., 1996). Ligated DNA was packaged using commercial extracts with size-selection (GigapackIII XL, Stratagene) according to manufacturer's instructions and transfected to XL1-Blue MRA *Escherichia coli* (Stratagene). Recombinant bacteria were plated onto agar at a density of 1000-2000 bacteria per plate. After growth at 37°C, the bacteria of each plate were pooled into 5 ml of LB medium. Subsequently, 4 ml was used for cosmid DNA isolation, while the remainder was kept as glycerol stock. The entire library consisted of $2.7x10^5$ clones in 180 pools representing 5.6 haploid genome equivalents based on an average insert size of 20 kb. Cosmid pools were screened by PCR with primers of the CT116 CAPS marker (Bonnema *et al*., 1997) and with degenerate primers which amplify LRR 1 to 17 of *Hcr9*s (HCR9C1F: 5'-catgggatggmrttsattgtgac-3' and HCR9C1R: 5'-catwgtgggattgtyccctcc-3'). Pools that yielded a PCR product were selected. To isolate single clones, $7.5x10^3$ bacteria of selected pools were screened for hybridisation with the two PCR products. Plasmid DNA of selected bacteria was isolated for further analysis.

λ-BlueStar library construction and screening

Genomic DNA from 4-week-old leaves of the breeding line Ontario 7518 (Cf18) showing *Cf-Ecp2*-mediated resistant (Laugé *et al.,* 1998a) was isolated according to the protocol described of Van der Beek *et al*. (1992). DNA was partially digested with *Sau*3A I to an average size of 40 kb and size- fractionated on a 10%-40% sucrose gradient (Sambrook *et al.,* 1989). Fractionated insert DNA with an average size of 20 kb was ligated at 4°C for 16 hr with 0.5µg of *Bam*HI-digested, dephosphorylated λBlueStar arms (Novagen). Ligated DNA was packaged using commercial extracts with size-selecting features (GigapackIII XL, Stratagene) according to manufacturer's instructions. Phages were transfected to host strain ER1647 (Novagen). The entire library consisted of $1.1x10^6$ phages representing 16 haploid genome equivalents based on an experimental average insert size of 15 kb. Phages were screened by hybridisation with the CT116 probe and a *Cf-9* probe covering the entire gene. Selected phages were automatically subcloned into plasmid by Cre-mediated excision from λBlueStar in host strain BM25.8 (Novagen). Plasmid DNA of selected bacteria was isolated for further analysis.

Restriction-mediated PCR fingerprinting and contig construction

To produce clone-specific markers, a restriction-mediated PCR fingerprinting method was optimised. Basically, the protocol consists of four steps: (1) digestion of plasmid DNA with two restriction enzymes; (2) ligation of matching adapters to sticky ends (3) pre-amplification and (4) labelled amplification which allows size-separation on sequencing type gels. For both plasmid- and cosmid library clones, 50 ng DNA was digested with *Eco*RI or *Apo*I and *Mse*I at 37°C. Simultaneously, adapters compatible to the restriction site were ligated. The *Eco*RI-adapter, which is also compatible to the *Apo*I restriction site, originated from the AFLP-protocol (Vos *et al*., 1995), the adapter compatible to the *Mse*I site was adapted from the Universal GenomeWalker kit (Clontech, Palo Alto, CA). PCR amplification was essentially performed according to the standard AFLP protocol (Vos *et al*., 1995) on ten times diluted restriction-ligation mixture. E-1_EX (5'-ctcgtagactgcgtaccaatt-3') and the AP1 (5'- taatacactcactatagggc-3') were used as primer set. A second, nested PCR using an fluorescently labelled, internal primer AP2 (5'-$_{IRD700}$actatagggcacgcgtgga-3') in combination with the E-1_EX-primer were performed on 25 times diluted amplification product of the first PCR. Samples were denatured and separated on a 5.5% polyacrylamide sequencing type gels using LI-COR Global IR^2 Systems. The presence or absence of polymorphic bands was scored by visual interpretation of outputs of the LI-COR system using the image interpretation software CROSSCHECKER (Buntjer, 2000; http://www.dpw.wau.nl/pv/pub/CrossCheck/download.html). The resulting binary data set was subsequently used for clustering by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using the software package NTSYSpc2.0 (Applied Biostatistics Inc.). UPGMA is the simplest method of distance tree construction. The calculated phylogenetic tree should represent contiguous clones clustered in clades. To confirm the position of each clone in a clade, fingerprinted samples were re-loaded on a gel in the order as indicated in the phylogenetic tree. A combination of restriction mapping, PCR analysis and DNA hybridisation resulted in additional data used for accurate aligning of library clones into a single contig. The origin of *Hcr9*s located on a library clone was identified by *Hinf*I, *Ava*II or *Taq*I digestion of the PCR amplified region of *Hcr9*s containing LRR 1 to 17 (primers: HCR9C1F/-R) and subsequently size-separation on 1.5% agarose gel.

Sequencing and sequence analysis

Selected library clones were sequenced by shotgun sequencing or by transposon-based sequencing using the GPS-LS linker scanning system (New England Biolabs). Greenomics (Wageningen, The Netherlands) performed sequencing of pBlueStar library clones. Evaluation of sequencing data and construction of sequence contigs was performed with Lasergene (DNASTAR Inc., Madison, WI, USA) software packages. PCR products selected to be sequenced were subcloned to pGEM-T Easy (Promega) or PCR-Script (Stratagene). Sequencing of these constructs and insequencing of the insert was done by BaseClear (Leiden, The Netherlands). DNA sequence similarity analysis was performed using BLASTN and BLASTX (Altschul *et al*., 1997). Promoter regions and polyA signal sites were analysed with the GeneBuilder prediction program (http://l25.itba.mi.cnr.it/~webgene/genebuilder). Protein structure predictions were performed using publicly available programs (http://www.expasy.ch/tools), signal peptide motif was identified by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP). The sequence of the *Cf-Ecp2 Hcr9* gene cluster and the orthologous *Hcr9* sequences have been deposited in the GenBank database (accession no. AY639601..AY639604)

Identification of orthologous *Orion Hcr9*s

Orthologous *Hcr9* genes were PCR-amplified using *OR-Hcr9*-specific primers sets with *Pfu-Turbo* proofreading DNA polymerase (Stratagene) at T_m=50°C, 30 cycli, using genomic DNA of tomato haplotypes

Cf0 (MoneyMaker), Cf-Ecp2 breeding line Ontario 7518 (Cf18), Cf-Ecp3 (*L. esculentum* G1.1153), and Cf-Ecp5 (*L. esculentum* G1.1161) and selected Cf-Ecp2, Cf-Ecp3 or Cf-Ecp5 genomic library clones as template and an extension time of 3.5 minutes at 72°C. Tomato Cf-Ecp3 and Cf-Ecp5 genomic libraries were donated by Y.Yuan and F. Meijer-Dekens, respectively (Wageningen University, The Netherlands). Positions of the primer sets are depicted in Figure 5. The forward primers were located at the first 30 nucleotides of a specific *Hcr9* (OR2ABf1: 5'-atgggttacgtaaaacttgtttttttaatg-'3, OR2Cf: 5'-atgggctacgtagaccttgtatttttttatg-3'), the reverse primer were located at the gene specific 3' untranscribed region (utr) (OR2A-utr: 5'-ctaatgcttttattacttagggaaatgcac-3', OR2B-utr: 5'-atagagattaagttgaatacctggagg-3', OR2C-utr: 5'-gaaaaatatcaagttgaatacctggag-3'). Partial *Cf-Ecp3 OR* sequences (Y. Yinan, unpublished data) were aligned to optimise primer sequences. PCR-amplification products were cloned into pGEM-T (Promega) and DNA sequencing was performed by BaseClear (Leiden, The Netherlands). Two independent clones per PCR-product were completely sequenced to avoid base pair changes introduced during PCR. To verify the physical linkage of OR2C orthologous genes with the CT116 CAPS marker, a PCR analysis was performed using the forward primer ORCutr-f, located at the 3'utr region an *OR2C* orthologue (ORCutr-f: 5'-aacctccaggtattcaacttg-3') and reverse primer in the CT116 CAPS locus (CT116F2r 5'-ttaccttctcaatcggcctcg-3'). PCR-amplification was performed on 200ng of genomic DNA or 20 ng of plasmid DNA in 25µL reaction volume using *Supertaq* (HT Biotechnology) DNA polymerase at annealing temperatures of 48°C and an extension time of five minutes at 72°C. PCR-amplification products were checked for size on a 0.8% agarose gel.

ACKNOWLEDGEMENTS

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Chapter 3

A resistance gene analogue fingerprint method facilitating mapping, cloning, and mRNA profiling of tomato *Cf* genes

This chapter is submitted for publication to *Molecular Breeding*.
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ABSTRACT

Resistance against the tomato fungal pathogen *Cladosporium fulvum* can be mediated by *Hcr9*s (Homologues of the *C. fulvum* resistance gene *Cf-9*). To support allele mining, mapping, cloning and mRNA profiling of tomato *Hcr9* genes, we developed a resistance gene analogue (RGA) fingerprint method to generate novel *Hcr9*-specific markers. The presence of both conserved and variable sequence domains in *Hcr9*s was exploited using a combination of PCR amplification and subsequent digestion. By the development of a fluorescent end-labelling method for restriction fragments, referred to as A/T labelling, high-resolution size-separation and detection of the complex RGA fingerprint pattern with a LI-COR automated sequencer became possible. The RGA fingerprint method was validated by the analysis of homozygous near-isogenic *Cf* lines and the segregation of *Cf-Ecp2* resistance gene candidates at the *Orion* (*OR*) locus. We identified several RGA-markers cosegregating with *Cf-Ecp2* resistance that corresponded to the three *Hcr9*s that are located at the *OR* locus. In addition, the results indicate that the *Hcr9* RGA fingerprint method facilitates the discrimination of highly homologous genes for the analysis of a mapping population. Finally, the *Hcr9* RGA fingerprint method was applied to study the expression of candidate *Cf-Ecp2* genes and showed that two out of the three *OR Hcr9*s were expressed *in planta*.

INTRODUCTION

The fungal pathogen *Cladosporium fulvum* is the causal agent of tomato leaf mold disease. In wild species of tomato, many accessions have been identified that contain resistance genes to *C. fulvum*. Breeders have introgressed *C. fulvum* resistance genes (designated *Cf*-genes) into commercially grown cultivars that provide protection against the fungus. The tomato - *C. fulvum* interaction has a gene-for-gene basis and the underlying molecular mechanism has been studied in detail (Joosten and De Wit, 1999). Cf-mediated recognition of the numerous Avr factors produced by *C. fulvum* evoke defence responses mostly including the hypersensitive response (HR) that is accompanied with a complete inhibition of fungal growth.

Cf resistance genes have been mapped at four different loci: (*i*) *Cf-2* and *Cf-5* are closely linked on Chromosome 6 (Dixon *et al.,* 1996, 1998) (*ii*) *Cf-4/ Cf4E*, and *Cf-9* are located at the short arm of Chromosome 1 at the *Milky Way* (*MW*) locus (Balint-Kurti *et al.,* 1994; Takken *et al.,* 1998; Van der Beek *et al.,* 1992), (*iii*) *Cf-Ecp5* is located at the *Aurora* (*AU*) locus, four centiMorgan (cM) proximal to the *MW* cluster (Haanstra *et al.,* 2000), and (*iv*) *Cf-Ecp2* and *Cf-Ecp3* are located at the *Orion* locus (*OR*), eight cM proximal to the *AU*

locus (Haanstra *et al.,* 1999; Yuan *et al.,* 2002). The *Cf* genes *Cf-9* (Jones *et al.,* 1994), *Cf-2* (Dixon *et al.,* 1996)*, Cf-4* (Thomas *et al.,* 1997), *Cf-4E* (Takken *et al.,* 1998), and *Cf-5* (Dixon *et al.,* 1998) have been cloned. The genes encode membrane-anchored, cytoplasmic glycoproteins. The extracellular domain mainly consists of leucine-rich repeats (LRRs) which are predicted to be directly or indirectly involved in the interaction with matching fungal elicitor proteins. Based on their sequence homology the known *Cf* genes belong to two families of genes. The first class is referred to as *Hcr9s* (Homologues of the *C. fulvum* resistance gene *Cf-9*) and the second (*Cf-2* and *Cf-5*) as *Hcr2*s (Homologues of the *C. fulvum* resistance gene *Cf-2*). Depending on the tomato genotype the *MW* locus can contain up to five *Hcr9*s. The short arm of Chromosome 1 harbours additional *Hcr9* clusters, so called *Northern Lights* (*NL*) and *Southern Cross* (*SC*) (Parniske *et al.,* 1999), but they do not contain *Cf* genes involved in resistance to *C. fulvum*. The overall organisation of these three clusters is depicted in Figure 1. RFLP analysis with a *Cf-9* probe demonstrated that the *AU* and *OR* loci with the *Cf-Ecp* genes, also contain *Hcr9*s (Haanstra *et al.,* 1999; Haanstra *et al.,* 2000; and Yuan *et al.,* 2002). 19 *Hcr9*s have been

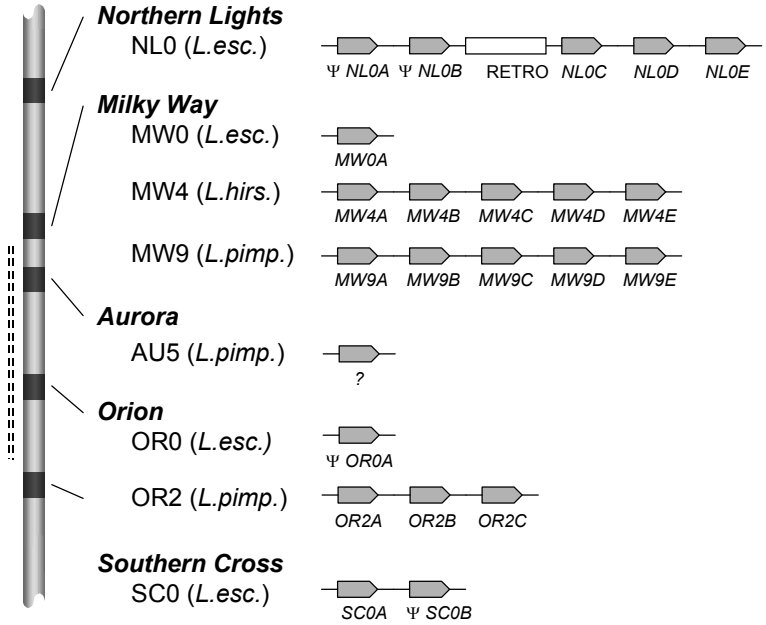

Figure 1. Schematic representation of the *Northern Light*s (*NL*), *Milky Way* (*MW*), *Orion* (*OR*) and *Southern Cross* (*SC*) clusters of *Hcr9*s on top of tomato Chromosome 1. On the left, the relative locations of the five *Hcr9* loci is presented. The dashed line indicates the *L. pimpinellifolium* introgression segment containing the *Cf-Ecp2* resistance gene. On the right, the physical organisation of *Hcr9*s is shown. Arrowed boxes indicate the position and the transcriptional polarity of *Hcr9*s. For the *MW* cluster, the organisation of three haplotypes is known (*L. esculentum, L. hirsutum*, and *L. pimpinellifolium*). For the *OR* cluster, the organisation of two haplotypes is indicated (*L. esculentum*, and *L. pimpinellofolium*). The organisation of the *AU* locus is unknown. *Hcr9* pseudogenes are labelled with a Ψ prefix. RETRO denotes a retrotransposon insertion in the *NL* haplotype.

sequenced to date. Comparison of the *Hcr9* sequences shows that sequence polymorphisms are generally present in the region encoding the N-terminal LRRs (Thomas *et al.,* 1997; Van der Hoorn *et al.,* 2001b).

The *Cf-Ecp2* resistance gene originates from *Lycopersicon pimpinellifolium* (Laugé *et al.,* 1998a) and has been accurately mapped on Chromosome 1, closely linked to the Cleaved Amplified Polymorphic Sequence (CAPS) marker CT116 (Haanstra *et al.,* 1999). A map-based and homology-based cloning approach has revealed the organisation of the *OR* cluster and has identified three candidate *Cf-Ecp2* genes that may mediate resistance to *C. fulvum* (Figure 1) (De Kock *et al.,* CHAPTER 2). By PCR-based cloning using *OR* sequences to design gene-specific primers, an *OR* orthologue was identified in *L. esculentum* Cf-0, designated Ψ*OR0A* (Figure 1) that corresponds with the *cf-ecp2* allele (De Kock *et al.,* CHAPTER 2). Ψ*OR2A* is highly homologous to *OR2C* (99.7% sequence homology) and has a 10 bp deletion in the region encoding LRR 4.

Two genomic libraries were screened for clones containing *Hcr9*s and/or the CT116 CAPS marker. Alignment of selected clones resulted in five contigs. Based on contig-derived sequences, three contigs could be linked to the previously reported *Hcr9* clusters *NL*, *MW* and *SC*. The genomic location of the fourth contig, containing one *Hcr9*, could not be assigned but may belong to the *Au* locus. The fifth contig comprising three *Hcr9*s could be assigned to the *OR* locus based on the physical linkage with CT116 (Figure 1). However, the *Cf-Ecp2 OR* cluster could contain additional *Hcr9*s or *Hcr9* pseudogenes.

A method was developed (designated as *Hcr9* resistance gene analogue (RGA) fingerprint method) to analyse the complexity and organisation of the *OR Hcr9* cluster. Conserved and variable DNA sequence domains in *Hcr9*s were exploited to generate *Hcr9*-specific RGA markers. To perform the RGA-fingerprint method at the genome-level, a fluorescent labelling method was developed that facilitated the detection of RGA markers on a LI-COR DNA sequencer. RGA fingerprint analysis of an F_2 mapping population that segregates for *Cf-Ecp2* resistance is compared with the analysis of the *Cf-Ecp2 OR* contig. Here, we present the *Hcr9* RGA-fingerprint method that can be applied for mapping, cloning and mRNA profiling of tomato *Hcr9* genes.

EXPERIMENTAL PROCEDURES

General strategy

The RGA-fingerprint method applied on *Hcr9* genes consists basically of four steps (Figure 2): (*i*) PCR-amplification of the variable LRR domain; (*ii*) digestion of PCR-amplification products by a restriction enzyme; (*iii*) fluorescent labelling of restricted fragments (if high-resolution size separation is required), and (*iv*) size separation on agarose or polyacrylamide gels.

(*i*) PCR-amplification of the variable LRR domain

Three different types of template were used for the PCR-amplification of the variable *Cf* domain. (a) Genomic DNA of *L. esculentum* near-isogenic lines MM-Cf0, MM-Cf4, and MM-Cf18 (harbouring no resistance gene, the *Cf-4* or the *Cf-Ecp2* resistance locus, respectively). Additionally, genomic DNA of plants originating from an F_2 mapping population (MM-Cf0 x MM-Cf18) that segregates for *Cf-Ecp2* resistance (Haanstra *et al.,* 1999) was used in our analyses. Genomic DNA was isolated according to Van der Beek *et al* (1992). Eight individual plants per genotype were analysed by RGA fingerprinting. (b) *L. esculentum* MM-Cf18 library clones located in the *OR* contig harbouring one of the three *Cf-Ecp2* gene candidates (De Kock *et al.,* CHAPTER 2). (c) cDNA derived from susceptible MM-Cf0 and resistant MM-Cf18 tomato plants (harbouring the *Cf-Ecp2* resistance gene). Total RNA was isolated with Trizol Reagent (Life Technologies) from approximately 0.2 g fresh leaf material according to the suppliers protocol. The RNA preparation was treated with DNaseI and purified using Gene-elute total RNA purification columns (Sigma). Ten µg total RNA was used for first strand cDNA synthesis.

The strategy for PCR amplification of the variable LRR domain of *Hcr9*s is depicted in Figure 3, and was performed on 250 ng of genomic DNA, 10 ng of plasmid DNA or 50 ng of first strand cDNA in a 50 µL reaction volume using *Supertaq* DNA polymerase (HT Biotechnology) and a mixture of primers RGA-f, RGA-fd, RGA-r, and RGA-rd (20 pmol of each primer per reaction). Optimal PCR-amplification was obtained with a touch-down PCR program (annealing temperature 62°C, 12 cycles -0.7°C/cycle; followed by 29 cycles with annealing temperature 53°C; extension at 72°C for 90 sec). PCR amplification products were separated on a 1% agarose gel to analyse size and quantity.

(*ii*) Digestion of PCR-amplification products

Before digestion, 200 ng of PCR-amplification products generated from plasmid DNA, 1 µg from genomic DNA or 1 µg from cDNA, were precipitated by EtOH and dissolved in ten µL H_2O. To generate RGA markers, complete digestion of PCR-amplification products was performed in a 20 µL reaction volume containing 15 units of a frequent cutting restriction enzyme [e.g. *Hinf*I, *Taq*I (Life Technologies) or *Hpy*CH4IV (New England Biolabs)] for three hours according to manufacturer's instructions.

Figure 2. Schematic representation of the *Hcr9* RGA fingerprinting method at a gene- and genome-level. (**1a**) PCR-amplification of the variable domain of an *Hcr9.* (**2a**) Digestion of the PCR product with a frequent cutting restriction enzyme. (**4a**) Separation of digested fragments on an agarose gel to visualise *Hcr9* specific fingerprints at gene level. As template are used library clones containing gene *A* (lane 1, 2), library clones containing both gene *A* and *B* (lane 3, 4), and library clones containing gene *B* (lane 5, 6). (**1b**) PCR-amplification of the variable domain of *Hcr9*s located in a genome. (**2b**) Digestion of the pool of PCR products with a frequently cutting restriction enzyme. (**3**) Fluorescent labelling of restriction fragments. Fill-in of 5' sticky overhang and addition of a 3'-terminal deoxyadenosine by *Taq* polymerase (A-tailing). The A-tailed DNA fragment is subsequently ligated to an IRDye-labelled amidated T-adapter containing a 3' deoxythimidine overhang at the bottom strand. The position of the infrared phosphoramide (IRDye)-labelled nucleotide in the adapter is indicated with an asterisk (∗), the amine-group is indicated by NH_2. (**4b**) A/T-labelled DNA fragments are separated on a denaturing polyacrylamide gel to visualise *Hcr9* specific fingerprints on a genome level. Near-isogenic lines with specific *C. fulvum* resistance clusters and a mapping population segregating for *Cf-Ecp2* resistance were used as a DNA template. See Experimental Procedures for further details.

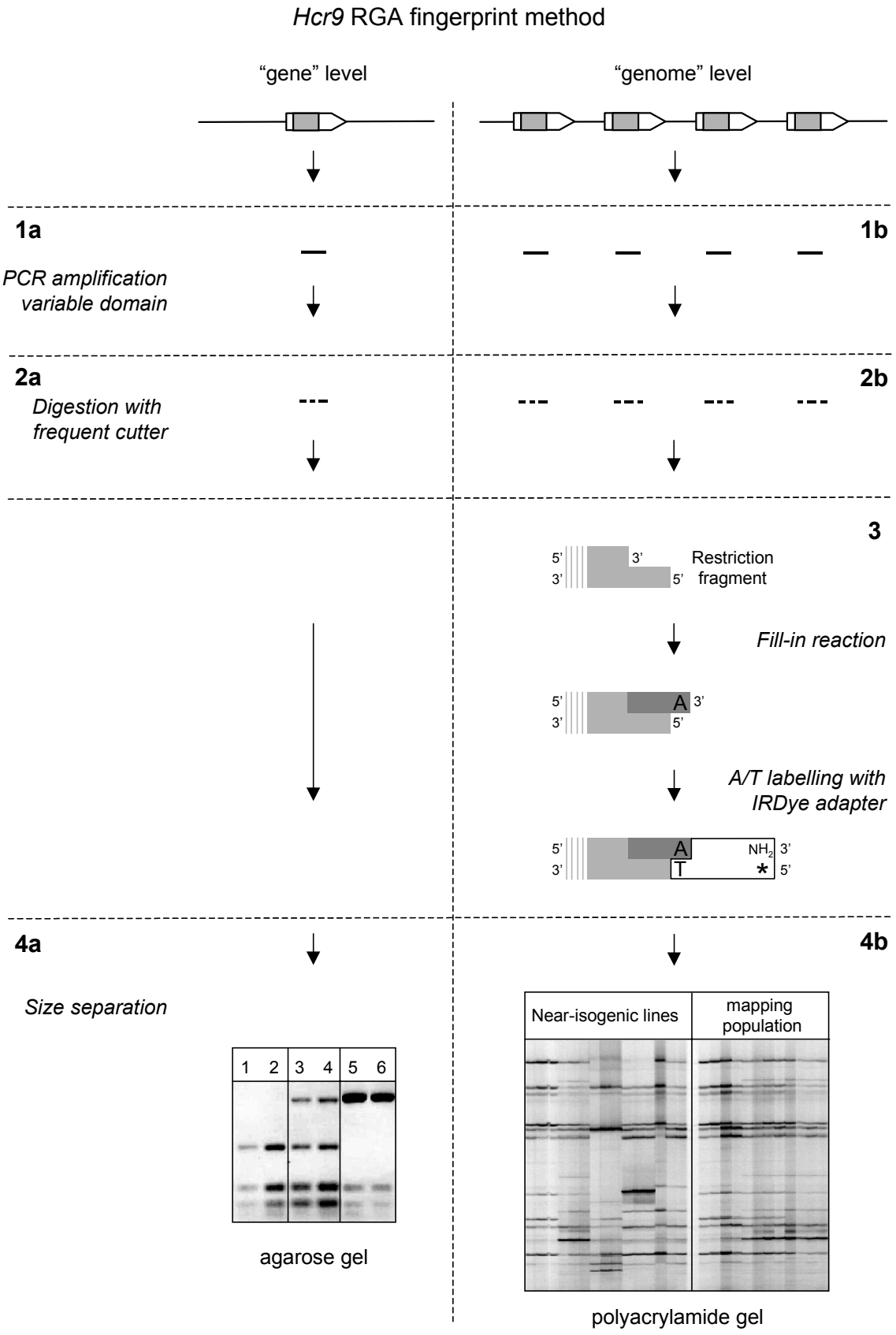
Hcr9 RGA fingerprint method
"gene" level
"genome" level
1a
1b
PCR amplification
variable domain
2a
2b
Digestion with
frequent cutter
3
5'
3'
3'
5'
Restriction
fragment
Fill-in reaction
A
A/T labelling with
IRDye adapter
T
NH2
*
4a
4b
Size separation
1 2 3 4 5 6
agarose gel
Near-isogenic lines
mapping
population
polyacrylamide gel

Figure 2.

(*iii*) Fluorescent labelling of restricted fragments

The digested products were size separated on a LI-COR 4200 DNA automated sequencer (LI-COR® Biosciences, Lincoln, NE) to visualise the complex pattern of RGA markers. DNA fragments were labelled with an appropriate IRDye to allow infrared detection during electrophoresis. We ligated an IRDye-labelled adapter to the restriction fragments making use of the principal of the pGEM®-T Vector systems (Promega).

The digestion mixture was precipitated with EtOH and 5' overhang sticky ends were filled-in with dNTPs at 70°C for 30 minutes (2.5 µmol dNTPs, 0.35 Units *Supertaq* polymerase, in a 1x PCR buffer; 5 µL total volume). By using a DNA polymerase without proofreading activity, a 3'-terminal deoxyadenosine is added during this reaction (referred to as A-tailing). Fragments generated after restriction by enzymes producing blunt ended DNA fragments were also subjected to this A-tailing. A-tailed DNA fragments were subsequently fluorescently labelled by the ligation to a T-adapter containing a 3'-terminal deoxythimidine and an IRDye™ label. The A-tailed DNA mixture (5 µL) was ligated O/N at 37°C to the IRDye- labelled T-adapter in a ligation mixture containing 1 Unit *T4 Ligase* (Invitrogen), 1 pmol IRDye-700 labelled T-adapter, 2 nmol

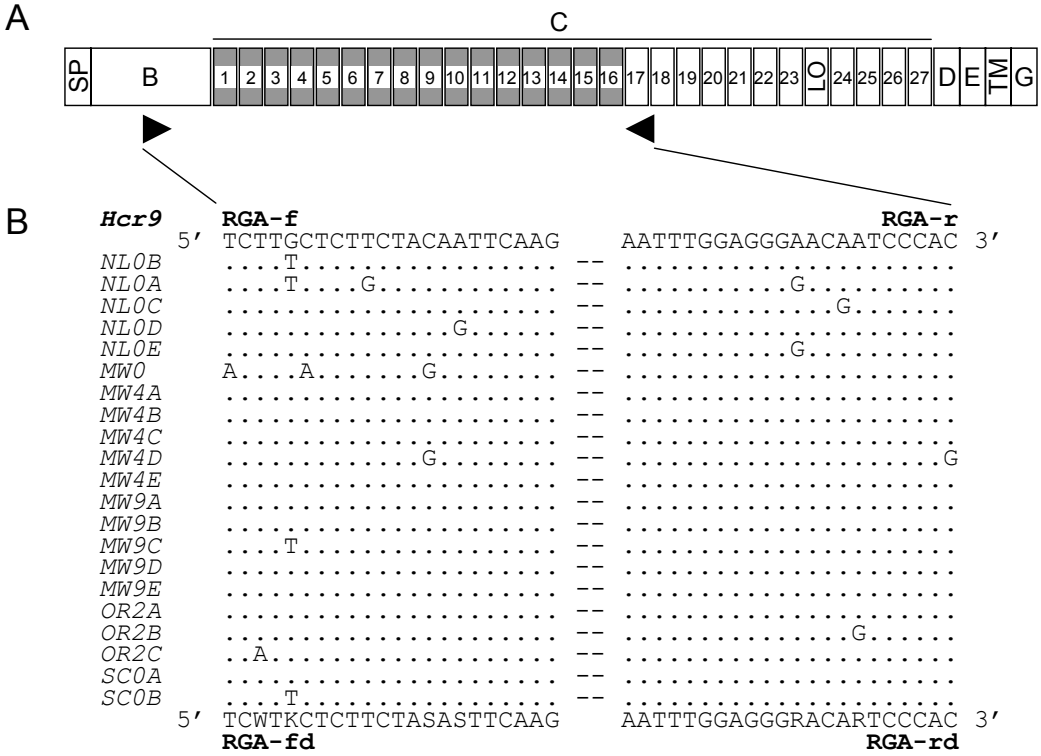

Figure 3. Primers designed to amplify the most variable LRR domain of *Hcr9* genes. (**A**) Schematic presentation of the protein domains present in an Hcr9: SP, signal peptide; B, cysteine-rich domain; C, domain containing 27 leucine-rich-repeats (LRRs), LRRs with high sequence polymorphism are in grey, the remaining LRRs display little sequence variation in the known *Hcr9*s, LO: Loop Out; D, domain without conspicuous features; E, acidic domain; F, putative transmembrane domain; G, basic cytoplasmic domain. Triangles indicate the location and orientation of the primers. (**B**) Sequence polymorphism of targeted sequences of known *Hcr9*s. The primer-annealing sequences of individual *Hcr9*s is aligned to create a consensus sequence (shown on the top). A dot (.) indicate identical sequence compared to consensus sequence. The primers RGA-f and RGA-r were designed based on the consensus sequence; the degenerate primers RGA-fd and RGA-rd were designed based on the polymorphisms in the consensus sequence (shown at the bottom). K = G or T; R = A or G; S = C or G; W = A or T.

ATP, 0.25 Units *Supertaq*, and 1.5 µL 5x T4-ligation buffer (Invitrogen) in a total volume of 10 µL. The T-adapter is generated by mixing equal amounts of the oligo's adT-top [$_{700}$GACTGCGTACCAATTCACT, near-infrared fluorescently labelled, (Biolegio, The Netherlands)] and adT-bot (^{P}GTGAATTGGTACGCAGT$_{NH2}$). The bottom strand (adT-bot) contains a 5'-terminal phosphate group for efficient ligation and a 3'-terminal amine group to avoid A-tailing of the adapter.

(*iv*) Size separation

Depending of the complexity of the RGA marker pattern, the RGA fragments were analysed by size separation on a 1.5% TAE agarose gel, or on a LI-COR 4200 automated DNA sequencer, essentially following the method published by Myburg *et al*. (2001).

RESULTS AND DISCUSSION

General strategy of *Hcr9* RGA-fingerprinting

(*i*) PCR-amplification. Most of the *Hcr9* genes encode proteins with 27 LRRs of which the N-terminal LRRs have high sequence polymorphism. Deletions in the B-domain occur in the *Hcr9* genes *NL0B, NL0D, MW4B, MW4D* and *MW4E*, whereas *Hcr9* genes *NL0E, MW4D* (= *Cf-4*) and *SC0B* have C-domains with less LRRs. Based on the known *Hcr9* sequences specific and degenerate primers were designed by which the variable LRR domain of all known *Hcr9*s could be amplified (Figure 3). PCR-amplification with a plasmid carrying an *Hcr9* as template resulted in a product of 1.1 up to 1.4 kb (Figure 2.1a). PCR-amplification on genomic DNA resulted in a mixture of PCR products comprising the variable domains of all *Hcr9*s in the genome (Figure 2.1b).

(*ii*) Digestion of PCR-amplification products. Most *Hcr9*-derived PCR products have a similar sizes and overlap upon size separation (results not shown). To generate *Hcr9* specific fragments, *Hcr9*-derived PCR products were subsequently digested. Based on the sequence of the known *Hcr9*s, the restriction enzymes *Hinf*I, *Taq*I, and *Hpy*CH4IV should discriminate between polymorphic sites of *Hcr9*s. Digestion of PCR-amplification products derived from a simple template DNA resulted in a fingerprint pattern in which gene-specific RGA markers could be identified (Figure 2.2a and 2.4a). Digestion of a pool of PCR-fragments from genomic DNA and subsequent size separation on agarose gel resulted in a diffuse smear of bands up to approximately 0.7 kb (results not shown). Size separation of this complex mixture of restriction fragments on a polyacrylamide gel was needed to distinguish individual restriction fragments.

(*iii*) Fluorescent labelling of restricted fragments and (*iv*) size separation. DNA fragments were fluorescently labelled enabling infrared detection upon separation on denaturing polyacrylamide gels with a LI-COR 4200 DNA automated sequencer (Figure 2.3). The use of IRDye-labelled primers for PCR-amplification was not possible since only the 5'- and 3'-

termini of the digested PCR products would be visualised, and internal restriction fragments would remain undetected. Therefore, an IRDye-labelled adapter was ligated to restriction fragments. Ligation of a PCR fragment into the pGEM®-T Vector is based on the presence of a single 3'-T overhang at the insertion site which provides a compatible overhang for PCR products. Thermostable DNA polymerases, like *Taq* polymerase, usually add a single deoxyadenosine to the 3'- ends of amplified fragments. The addition of an adenosine (referred to as A-tailing) to restriction fragments with *Taq* polymerase will therefore result in compatible ends for the ligation to an (IRDye fluorescently labelled) adapter containing the 3' T-overhang (Figure 2.3). Blocking of the 3' end of the adapter with an amine (NH_2) group should prevent extension at the 3' end resulting in unwanted A-tailing of the T-adapter. By this new fluorescent labelling method, referred to as A/T labelling, a complex DNA mixture can be labelled with a fluorescent dye that allowes high-resolution size separation of restriction fragments and detection with a LI-COR 4200 DNA automated sequencer.

The A/T labelling method was optimised using a complex DNA mixture generated by restriction-mediated PCR fingerprinting of library clones (De Kock *et al.,* CHAPTER 2). Figure 4, panel A, shows a PCR fingerprint pattern of five contiguous clones using an IRDye-labelled primer set. In panel B, identical fingerprints are shown which were generated from the same contiguous clones by PCR-amplification using non-labelled primers and subsequently labelling the amplification products using the A/T labelling

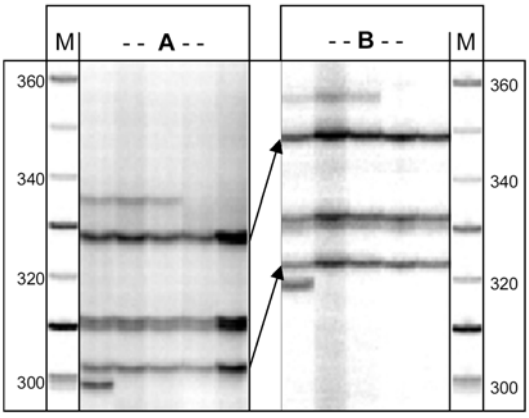

Figure 4. Size separation of fluorescently labelled DNA samples. For the cloning of *Cf-Ecp2*, library clones were isolated and aligned into contigs by restriction-mediated PCR fingerprinting followed by contig calculation (De Kock *et al.,* CHAPTER 2). **(A)** A complex, fluorescently labelled mixture generated by restriction-mediated PCR fingerprinting of contiguous library clones using IRDye-labelled primers. (**B**) A complex DNA mixture generated by restriction-mediated PCR-fingerprinting of contiguous library clones using non-labelled primers. The PCR mixture was subsequently labelled using the IRDye-labelled T-adapter. The fluorescently labelled fingerprinting pattern is shifted 19 bp upwards compared with the fingerprint pattern presented in A (indicated by arrows). Each lane represents the DAN fingerprint of one individual library clone; M: marker, sizes in base pairs.

method. The resulting fingerprint pattern is shifted 19 bp upwards compared with the original fingerprint pattern due to the presence of one adapter (size 19 bp) in each PCR product. Surprisingly, both T4 ligase and DNA polymerase are required for ligation of the adapter to the A-tailed DNA fragments (data not shown). Very efficient labelling using an excess of label and a simple PCR product results in the addition of two adapters, one to each side of the PCR product (results not shown).

A-tailing is an essential part for successful A/T labelling as incomplete addition of an adenine nucleotide to the restriction fragments affects the labelling efficiency. It has been reported that certain terminal nucleotides can either inhibit or enhance adenine addition by DNA polymerase (Magnuson *et al.,* 1996). However, the sequences of the restriction site overhangs did not influence the labelling efficiency.

Fluorescent labelling may also be applied in microsatellite analysis. This type of genetic marker often produces a complex mixture of PCR products that requires high-resolution separation on polyacrylamide gels. In the optimisation step of micro-satellite analyses, only a fraction of the tested primer combinations lead to informative PCR products. Indeed, extensive microsatellite analyses could be optimised by fluorescent labelling of unlabelled PCR-mixtures followed by size-separation on polyacrylamide gels (T. Marcel, unpublished data). Subsequently, only primers combinations that were found to be informative were fluorescently synthesised and used for high throughput applications.

Automated sequencers are widely used for DNA sequencing, microsatellite analysis, AFLP® analysis and reverse genetics. These sequencers are perfectly suited for the high-resolution size separation, detection and analysis of PCR products. We anticipate that the A/T labelling procedure can also be applied with other fluorescent dyes. If an automated sequencer is not available, radio-isotopic labelling of restriction fragments can be considered by filling in radioactive dNTPs to enable visualisation on an autoradiogram. A/T labelling can then be omitted.

Application of RGA fingerprinting on plasmid DNA

For the cloning of *Cf-Ecp2*, genomic library clones containing *Hcr9*s were isolated and the genomic DNA inserts were aligned into contigs. Application of the RGA fingerprint method on library clones harbouring an *Hcr9* resulted in unique, gene-specific *Hcr9* fingerprint patterns (Figure 2.4A). Additionally, RGA-fingerprint patterns of known *Hcr9*s were predicted based on their sequence and were compared with experimental fingerprint patterns. Subsequently, *Hcr9* contigs covering known *Hcr9* clusters could be identified and were assigned to the *NL*, *MW* and *SC* locus (results not shown). The contiguous clones overlapping with the CT116 CAPS marker contained in total three *Hcr9*s with specific RGA-fingerprinting patterns.

So far, it was unknown whether the constructed contig with three *Hcr9*s completely covered the *Cf-Ecp2* cluster at the *OR* locus. Possibly, screening of the genomic library was incomplete and some *OR Hcr9*s were missing. Therefore, the RGA-fingerprinting method was optimised to enable the fingerprinting of *Hcr9* genes from genomic DNA.

Application of RGA fingerprinting on genomic DNA

The RGA-fingerprint method on genomic DNA, it was first applied on near-isogenic lines (NILs) MM-Cf0, MM-Cf4 and MM-CfEcp2 for validation. Since the sequences of *Hcr9*s present in the *NL*, *MW*, and *SC* clusters is known (Parniske *et al.,* 1999), it was possible to predict the RGA-fingerprint pattern of MM-Cf0 and MM-Cf4 genotypes by computational analysis. The main difference between MM-Cf0 and MM-Cf4 is the number of *Hcr9*s at the *MW* cluster: MM-Cf0 contains one *Hcr9* while MM-Cf4 has five *Hcr9*s. The observed RGA-fingerprint patterns of the Cf0- and Cf-4 NILs were similar to those predicted by computational *Hcr9* RGA fingerprinting (results not shown). In subsequent repeat experiments, the *Hcr9* RGA fingerprinting method was found to be reproducible.

Genomic DNA of the near-isogenic line MM-Cf-Ecp2 gave a specific *Hcr9* RGA fingerprint with fragments that were both different in size and quantity from those observed for MM-Cf0 and MM-Cf4. Additional fragments that cosegregated with *Cf-Ecp2* resistance must be amplified from the *OR* cluster and possibly from the *Cf-Ecp2* gene itself. This was verified by the analysis of a mapping population segregating for *Cf-Ecp2* resistance. Eight individual plants with an rr (*cf-ecp2*,*cf-ecp2*), Rr (*Cf-Ecp2*,*cf-ecp2*), or RR (*Cf-Ecp2*,*Cf-Ecp2*) genotype were selected based on previous genetic analysis (Haanstra *et al*., 1999). If the *OR* contig covers the complete *OR Hcr9* cluster, all *OR*-specific genomic RGA markers should be covered by markers from the library clones of the putative *OR* contig.

RGA-fingerprinting of individual plants of the mapping population resulted in three types of fragments (Figure 5a): (*i*) the majority of fragments are present in both the resistant and susceptible genotypes. These fragments are derived from *Hcr9*s located in the *NL*, *MW*, *AU* and *SC* clusters that are present in both the susceptible MM-Cf0 tomato genotype and the resistant Cf18 genotype. In addition, these fragments may be derived from conserved sequences present in different *Hcr9*s. (*ii*) Fragments that cosegregate with resistance and are in coupling phase. Seven *Cf-Ecp2*-linked markers were generated using the restriction enzyme *Hinf*I of which three important markers are indicated in Figure 5a. Application of the restriction enzymes *Taq*I and *Hpy*CH4IV resulted in additional markers (results not shown). Importantly, all *Cf-Ecp2* linked RGA-markers could be reproduced from genomic library clones containing the candidate *Cf-Ecp2* gene *OR2-A, OR2-B*, or *OR2-C* (Figure 5b). Based on the complete correlation of the genomic RGA-fingerprint with the *OR*-contig RGA-fingerprint, we concluded that the contig completely covers the *Hcr9* cluster and the *Cf-Ecp2 OR* gene cluster contains not more than three

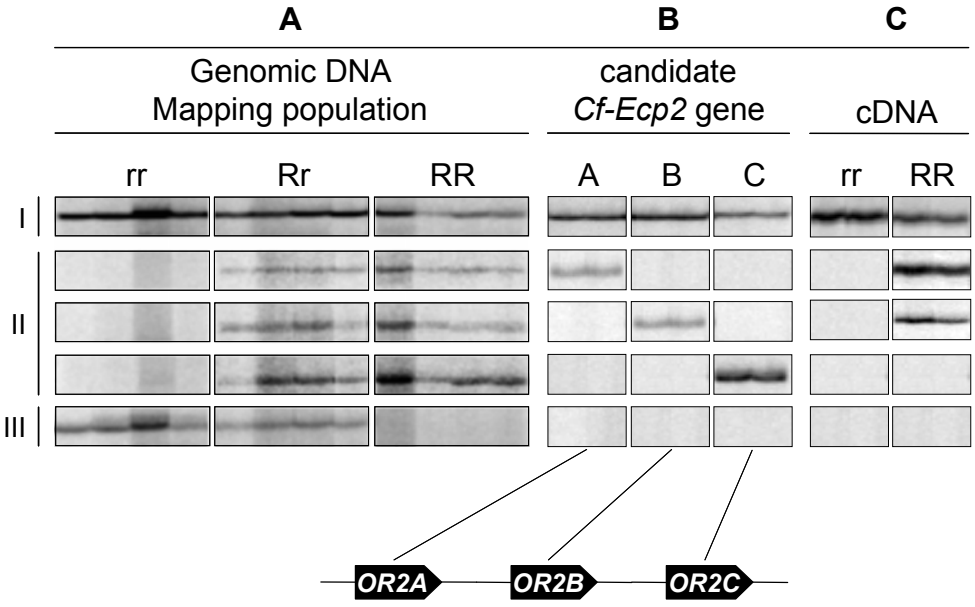

Figure 5. Co-segregation of RGA-based markers with *Cf-Ecp2* mediated resistance and candidate *Cf-Ecp2* genes at the *Orion* (*OR*) *Hcr9* cluster. (**A**) Application of *Hcr9* RGA-fingerprinting method on a mapping population segregating for *Cf-Ecp2* resistance: rr: homozygous susceptible (*cf-ecp2*,*cf-ecp2*); Rr: heterozygous resistant (*Cf-Ecp2*,*cf-ecp2*); RR: homozygous resistant (*Cf-Ecp2*,*Cf-Ecp2*). Three types of RGA-markers were identified: Type I: markers present in all genotypes, type II: markers cosegregating with the *Cf-Ecp2* allele, type III: markers linked the *cf-ecp2* allele. (**B**) *Hcr9* RGA-fingerprinting applied on individual *Cf-Ecp2* gene candidates located in the *OR* gene cluster. All type II markers were identified in one of the three candidate *Cf-Ecp2* genes (**C**) *Hcr9* RGA-fingerprinting applied on cDNAs derived from susceptible (rr) and resistant (RR) Cf18 plants. Note that of the *Cf-Ecp2* gene candidates only *OR2A* and *OR2B* are transcribed in resistant *Cf-Ecp2* plants.

*Hcr9*s. (*iii*) A third class of fragments was identified which was in repulsion phase with *Cf-Ecp2*. This single RGA marker was derived from the orthologous gene Ψ*OR0A,* located in the *OR* locus of MM-Cf0 (De Kock *et al.,* CHAPTER 2). The deletion of 10bp in Ψ*OR0A* compared to its orthologous gene in *Cf-Ecp2*, *OR2C*, resulted in a 10 bp shift of the corresponding *Hcr9* RGA marker. This data indicate that the *Hcr9* RGA fingerprint method facilitates the discrimination of highly homologous genes in the analysis of a mapping population.

We additionally conclude that the RGA fingerprint method is a versatile tool for allele mining of *Hcr9*s. Little is known about the diversity of *Hcr9* genes and *Hcr9* clusters in wild species. Throughout the *Lycopersicon* genus, species were recently identified that specifically respond with HR to the Avr4 and Avr9 proteins of *C. fulvum* (M. Kruijt, *pers. comm*.; Van der Hoorn *et al.,* 2001a). The recognition in these species could be correlated with the presence of genes highly homologous to *Cf-4* and *Cf-9*. RGA fingerprint analysis of additional sets of *Lycopersicon* accessions should enable the correlation between Avr

responsiveness and resistance with particular *Hcr9* RGA markers. Linkage of *Hcr9* RGA markers can subsequently be an indication of clustering of particular *Hcr9* genes.

Resistance-gene-analogue mRNA profiling

In the previous section, we described the fingerprinting of *Hcr9*s and its application to facilitate the molecular isolation of *OR Hcr9* clusters. It would be an asset to identify which *Hcr9* genes are transcribed *in planta*. Therefore, the method was applied on total cDNA preparations from susceptible MM-Cf0 and resistant MM-Cf-Ecp2 plants. Focussing on the *Hcr9* RGA markers specific for the *OR Hcr9* cluster, cDNA-RGA markers were only observed for *OR2A* and *OR2B* (Figure 5c). In resistant *Cf-Ecp2* plants, transcripts of *OR2C* were not detected. Subsequent RT-PCR analysis using primer sets specific for each *OR2* paralogue confirmed these results (results not shown). Apparently, only *OR2A* and *OR2B* are candidate genes for *Cf-Ecp2*.

Advantages and limitations of the *Hcr9* RGA fingerprint method

In general, *R* proteins contain various domains involved in pathogen recognition and a subsequent disease resistance response. Methods to identify resistance gene analogues are based on the presence of homologous regions that can be a target for probe hybridisation in RFLP analysis of populations segregating for resistance. Alternatively, gene- or domain-specific primers can amplify novel *R*-genes. Presence of polymorphic sites between gene analogues can further be exploited to visualise all members of a gene family.

Many *R* gene identification methods have been described. Detection of *R* gene homologues by RFLP analysis is reasonably robust but the resolution is rather low. *Cf* gene loci have been studied by RFLP analysis using *Cf*-derived probes (Van der Beek *et al.,* 1992; Jones *et al.,* 1993; Dickinson *et al.,* 1993; Balint-Kurti *et al.,* 1994, Haanstra *et al.,* 1999; Yuan *et al.,* 2002). Another class of *R* genes, the *Pto* kinase family, has been studied in great detail by a combination of RFLP, PCR-amplification, and sequence analysis (Riely *et al.,* 2001; Vleeshouwers *et al.,* 2001). The majority of the *R* genes contain leucine-rich repeats (LRRs) and nucleotide binding site (NBS) domains, the so-called NBS-LRR class of *R* genes. Sequence homology between NBS-LRR analogues is lower than within classes of *Cf*- or *Pto* homologous. Therefore, variation in NBS-LRR genes is generally studied by PCR using specific primers and individual *R* gene analogues are identified based on PCR product size differences (Chen, *et al.,* 1998). This method has been used to identify molecular markers tightly linked to or cosegregating with disease resistance genes (Toojinda *et al.,* 2000; Shi *et al* 2001; Di Gaspero and Cipriani, 2003; Yan *et al.,* 2003).

An advantage of PCR-based detection is the ability to clone and to obtain sequence information of the *R* gene derived PCR products. Success of PCR-based methods depends fully on the compatibility and degeneracy of the primers and the resolution of size-separation. To increase resolution, PCR-amplification products can subsequently be used as template in a modified AFLP-reaction (Hayes, *et al*., 2000, Bakker *et al.,* 2003, Van der Linden, *et al*., 2004). Successful amplification of the individual members of a particular *R* gene cluster will depend on the specificity of the primers and the homology at the primer-annealing site (Bakker *et al.*, 2003).

Like most RGA-fingerprint methods described in literature, our method requires a PCR-amplification as the first step. The highly conserved sequences adjacent to a variable domain in individual *Hcr9* analogues was used for PCR amplification. However, it cannot be excluded that unknown *Hcr9*s with a lower homology are not amplified. Additionally, the *Hcr9* RGA fingerprint method is specific for *Hcr9*s, and does not include *Hcr2*s in the analysis. Primer sequences can easily be adapted for *Hcr2*s as these class of *R* genes also contain highly conserved sequences adjacent to the variable domain and have a similar domains structure.

Subsequent to the PCR amplification we exploited the polymorphisms present in the RGAs by restriction enzyme analysis. To visualise the complex RGA fingerprint profile, we developed a new labelling strategy, the A/T labelling method, which allowed detection of all labelled bands after high-resolution size separation. Other RGA-detection methods could exploit this strategy as well.

Finally, we successfully applied our method to verify the presence and number of *Hcr9*s in the *OR* cluster and the transcriptional activity of the *Hcr9*s. As alternative, expression of a single *Cf* gene can be analysed by gene-specific reverse transcriptase (RT) PCR (Panter *et al.,* 2002). A drawback of this method is the requirement a primer set specific for the *Cf* gene of interest. Aspecific annealing of the primer set to other *Hcr9*s can result in non-informative results. Moreover, expression analysis of individual genes requires separate experiments or multiplexed PCRs. Advantageous for the *Hcr9* RGA profiling method is the visualisation of the expression of a complete gene-family. Furthermore, genomic fingerprint patterns can easily be converted to the expression profile as is presented in Figure 5.

ACKNOWLEDGEMENTS

We thank Bas Brandwagt for critical comments on the manuscript.

Chapter 4

Functional analysis of candidate *Cf-Ecp2* genes shows that recognition of the *C. fulvum* Ecp2 elicitor is not solely mediated by an *Hcr9* gene

Co-autheurs: Bas F. Brandwagt, Pierre J.G.M. de Wit, and Pim Lindhout

ABSTRACT

Hcr9 genes (Homologues of the *Cladosporium fulvum* resistance gene *Cf-9*) often mediate resistance against the tomato fungal pathogen *C. fulvum* upon recognition of matching fungal avirulence gene products. The resistance gene *Cf-Ecp2* mediates the recognition of the virulence factor Ecp2 and is located in the *Orion* (*OR*) cluster on the short arm of tomato Chromosome 1. A map-based and homology-based cloning resulted in a contig covering the *OR* resistance locus and three *Hcr9*s were identified as candidate *Cf-Ecp2* genes. Here, we describe various methods to identify the functional *Cf-Ecp2* gene. Transient expression in *Nicotiana* species and complementation analysis in tomato were exploited to test candidate *Cf-Ecp2* genes for the ability to mediate Ecp2 recognition. Despite applying agroinfiltration and coplementation assays, we were not able to identify which of the three *OR Hcr9* represents the functional *Cf-Ecp2* gene. Based on these results we have to conclude that recognition of the *C. fulvum* Ecp2 elicitor is not solely mediated by an *OR-Hcr9* and an additional tomato-derived HR-stimulating factor is required for *Cf-Ecp2*/*Ecp2* mediated resistance.

INTRODUCTION

The fungal pathogen *Cladosporium fulvum* is the causal agent of tomato leaf mould disease. Resistance against *C. fulvum* can be mediated by the resistance genes *Cf-2*, *Cf-4*, *Cf-4E*, *Cf-5* and *Cf-5* which have been introgressed from wild *Lycopersicon* species into commercially grown tomato (*L. esculentum*). These genes are required for specific recognition of the fungal elicitor proteins Avr2, Avr4, Avr4E, Avr5 or Avr9, respectively, and for the triggering of a hypersensitive response (HR)-associated resistance (reviewed by Joosten and De Wit, 1999). *Cf-9*, *Cf-4*, and *Cf-4E* are located in gene clusters at the *Milky Way* (*MW*) locus on the short arm of Chromosome 1 of tomato. These are very homologous and are referred to as *Hcr9*s (Homologues of the *C. fulvum* resistance gene *Cf-9*). In addition, *Cf-2* and *Cf-5* are located in gene clusters on the short arm of Chromosome 6 and are referred to as *Hcr2*s (Homologues of the *C. fulvum* resistance gene *Cf-2*). Both *Hcr9*s and *Hcr2*s encode membrane-anchored, cytoplasmic glycoproteins of which the extracytoplasmic domain mainly consists of leucine-rich repeats (LRRs) which are predicted to be involved in the interaction with the matching fungal elicitor proteins (Jones *et al.,* 1994; Thomas *et al.,* 1997; Takken *et al.,* 1998). The short arm of Chromosome 1 harbours additional *Hcr9* clusters, such as *Northern Light* (*NL*) and *Southern Cross* (*SC*) (Parniske *et al.,* 1999), but these clusters do not contain functional *Cf* resistance genes. *Cf-Ecp2* and *Cf-Ecp3* are also located on the short arm of Chromosome

one at the *Orion* (*OR*) locus, closely linked to the CAPS marker CT116, twelve centiMorgan (cM) proximal to the *MW* locus (Haanstra *et al.,* 1999; Yuan *et al.,* 2002), while *Cf-Ecp5* is at the *Aurora* locus, four cM proximal to the *MW* cluster (Haanstra *et al.,* 2000). As the *Cf-Ecp2* gene mediates recognition of the virulence factor Ecp2, *Cf-Ecp2* was speculated to confer durable resistance against *C. fulvum* (Laugé, *et al*., 1997, 1998).

Map-based and homology-based cloning resulted in overlapping genomic DNA fragments covering the *OR* locus and CT116 (De Kock *et al*., CHAPTER 2). Three *Hcr9*s were identified as candidate *Cf-Ecp2* genes, designated *OR2A*, *OR2B*, and *OR2C* (Figure 1a). In addition, a gene encoding the 26S proteasome regulatory subunit S12 was located at the constructed contig. It was not expected that this gene, which is shows no cDNA polymorphisms in the tomato genus, is involved in the specific recognition of the *C. fulvum* elicitor Ecp2. The organisation of the isolated *OR Hcr9* cluster was compared with the genomic organisation of the *Cf-Ecp2 OR* cluster by a *Hcr9* resistance gene analogue (RGA) fingerprinting method (De Kock *et al*., CHAPTER 3). The results confirmed that the *Cf-Ecp*2 *OR* cluster indeed contains three *Hcr9* genes. The RGA fingerprint method also allowed to determine the expression of the individual candidate *Cf-Ecp2* genes. In

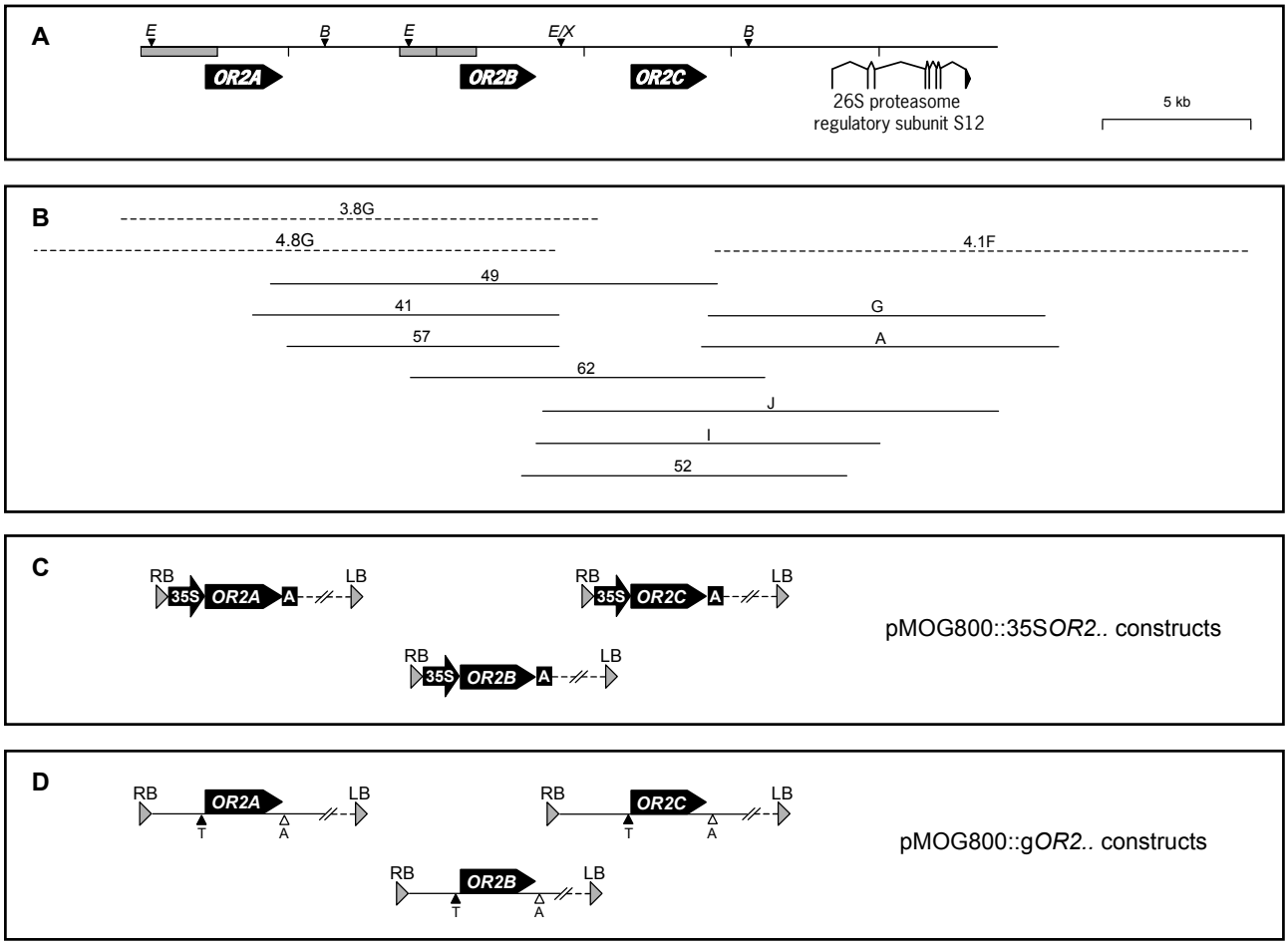

Figure 1.

resistant *Cf-Ecp2* plants, only *OR2A* and *OR2B* are transcribed (Figure 2a,b). Consequently, either *OR2A* or *OR2B* represents the functional *Cf-Ecp2* gene, whereas *OR2C* is most probably not involved as its transcription is absent or below the detection level.

Here, we present various attempts to reveal the functional *Cf-Ecp2* gene. Despite the clear differences in expression, all three *OR Hcr9*s were considered as candidate *Cf-Ecp2* genes. The *Agrobacterium*-mediated transient expression (agroinfiltration) in tobacco has facilitated successful expression of extracellular elicitors and membrane-anchored Cf-proteins (Van der Hoorn *et al.,* 2000). This method was used to investigate which of the three *Hcr9* genes responds with an HR through recognition of Ecp2. Tobacco (*Nicotiana tabacum*) responds naturally with an HR upon Ecp2 exposure (De Kock *et al.*, CHAPTER 5) and can therefore not be used for *Cf-Ecp2* agroinfiltration analysis. Alternative *Nicotiana* species suitable for agroinfiltration were used. In addition, complementation experiments were performed by stable transformation of candidate *Cf-Ecp2* genes to susceptible tomato and the subsequent functional analysis of transgenic progeny plants. Despite applying all commonly used functional assays, the functional *Cf-Ecp2* gene could not be identified. Based on the results we have to conclude that recognition of the *C. fulvum* Ecp2 elicitor is not solely mediated by an *OR-Hcr9* and possible explanations are discussed.

RESULTS

Functional analysis of candidate *Cf-Ecp2* genes by transient assays in *Nicotiana* spp.

The *OR* contig was constructed by clones isolated from a binary cosmid library (pCLD04541 vector, Dixon *et al*., 1996) and from a pBlueStar (Novagen) plasmid library

Figure 1. The *Cf-Ecp2 Orion* cluster and the constructs used for functional analysis of candidate *Cf-Ecp2* genes. (**A**) Physical map of the *Cf-Ecp2* locus. The position and orientation of three intronless *Hcr9* ORFs is indicated by arrowed boxes. The position of the 26S proteasome regulatory subunit S12 exons are shown by connected boxes and triangle. Restriction sites used for subcloning genomic *Cf-Ecp2* candidate genes are indicated by triangles: *E*, *Eco*RI; *B*, *Bam*HI; *X*, *Xho*I. A five-kb scale is shown by ticks. (**B**) Position of pCLD04541 binary cosmid inserts (dashed lines) and pBIVM2 inserts (solid lines) at the *Cf-Ecp2* locus that were used for agroinfiltration in *Nicotiana* species. (**C**) Schematic representation of T-DNA of pMOG800::35S*OR2* constructs with PCR-subcloned *Cf-Ecp2* candidate genes. Symbols: RB, right border of T-DNA; LB, left border of T-DNA, 35S, Cauliflower Mosaic Virus (CaMV) 35S promoter; A, potato proteinase inhibitor-II polyadenylation region. Dashed lines indicate flanking T-DNA sequence containing neomycin-phosphotransferase II cassette. (**D**) Schematic representation of T-DNA of pMOG800::g*OR2* constructs with subcloned *Cf-Ecp2* candidate genes flanked by genomic promoter and polyadenylation regions. Symbols: T, predicted TATA box; A, predicted polyadenylation region. Solid lines indicated the subcloned genomic region, dashed lines indicate flanking T-DNA sequence containing neomycin-phosphotransferase II cassette.

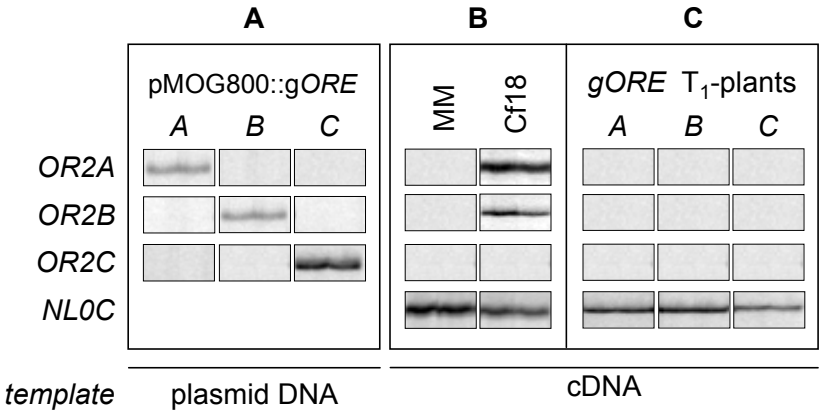

Figure 2. *Hcr9*-specific resistance-gene-analogue (RGA) fingerprint analysis to determine the expression of individual *Cf-Ecp2* candidate genes. (**A**) Gene-specific RGA markers derived from individual binary plasmid DNA used for stable transformation of tomato, harboring a genomic subclone of *OR2A*, *OR2B* or *OR2C*. (**B**) RGA markers specific for *OR2A* and *OR2B* are produced from cDNA of tomato Cf18 plants, carrying the *Cf-Ecp2* resistance gene. These genes are absent and therefore not transcribed in susceptible MoneyMaker (MM). Transcripts of *OR2C* were absent in tomato Cf18 plants or below detection level. Transcript-derived RGA markers from other expressed *Hcr9* genes, like *NL0C* were identified in both MM and Cf18 plants. (**C**) Transcripts for *OR2A*, *OR2B*, *OR2C* are absent or below detection level in primary transformant plants carrying one of the candidate *Cf-Ecp2* genes as RGA markers could not be produced. Expression of *NL0C* in these transgenic plants was comparable with the expression in MM and Cf18.

(De Kock *et al*., CHAPTER 2). Prior to finishing the consensus sequence and the annotation of the *OR* cluster, complete inserts of library clones were tested for mediating Ecp2 recognition by agroinfiltration. The physical location in the *OR* contig of the genomic clones used for agroinfiltration is indicated in Figure 1b. Inserts of pBlueStar clones were subcloned into a binary vector, pBIV2M (a pCGN1548 derivative, McBride and Summerfelt, 1990) to enable *Agrobacterium*-mediated transformation. Unfortunately, DNA-instability of the cosmids 3.8G and 4.8G was observed in *Agrobacterium* that resulted in recombination of the insert. This artefact precluded a straight forward functional analysis of *Cf-Ecp2* candidates with these binary cosmid clones.

Nicotiana species that are not responsive to Ecp2, and are suitable for agroinfiltration (*N. benthamiana, N. glutinosa*, *N. kawakamii*, *N. langsdorfii*, and *N. tomentosiformis*) were infiltrated with of *Agrobacterium* cultures carrying *Ecp*2 and candidate *Cf-Ecp2* genes. In all tested *Nicotiana* species, leaf sectors co-infiltrated with cultures expressing the control gene pairs *Cf-4*/*Avr4* or *Cf-9*/*Avr9* showed the expected full necrosis. Control leaf sectors infiltrated with cultures expressing the individual genes did not show any necrosis (results not shown). In the tested *Nicotiana* species, none of the genomic *Cf-Ecp2* candidates showed a specific HR upon co-agroinfiltration with an *Ecp*2-expressing culture.

Subcloning of candidate *Cf-Ecp2* genes into binary expression vectors became possible after completion of the *OR* sequence. The expression constructs with candidate *Cf-Ecp2* genes are depicted in Figure 1c. To include the possibility that *Cf-Ecp2* candidate genes act together in a complex, all possible combinations of the candidate *Cf-Ecp2* genes were assayed. However, in the tested *Nicotiana* species agroinfiltration assays with the single-, or combinations of candidate genes, did not show any specific HR upon co-infiltration with an *Ecp*2-expressing culture.

These results may suggest that an additional extracellular tomato component would be required for Ecp2 recognition. Therefore, apoplastic washing fluid of healthy- and *C. fulvum*-infected tomato leaves was co-infiltrated both during agroinfiltration, and three days post agroinfiltration. Additionally, to exclude insufficient Ecp2 production upon agroinfiltration, candidate *Cf-Ecp2* genes were transiently expressed in *Nicotiana* plants systemically overexpressing a secreted Ecp2 upon PVX::PR1a*Ecp*2 infection. Unfortunately, addition of tomato apoplastic proteins or PVX-mediated Ecp2 overproduction did again not result in clear *Cf-Ecp2* mediated HR responses.

Functional analysis of candidate *Cf-Ecp2* genes by transient assays in tomato

Agroinfiltration in tomato was optimised. The elicitors Avr4 and Ecp2 were used to induce a specific HR upon agroinfiltration in tomato plants containing *Cf-4* or *Cf-Ecp2*, respectively. Additionally, the gene-pair *Cf-4*/*Avr4* was used in the optimisation of co-agroinfiltration. Different parameters were varied like e.g. *A. tumefaciens* strain, buffer composition, culture density, plant age (detailed results not shown). In general, aspecific chlorosis and necrosis was frequently observed in the infiltrated sector of the tomato leaf, most likely induced by *Agrobacterium* cells (Figure 3a). We were not able to show Avr4- or Ecp2-induced HR responses upon agroinfiltration in tomato plants with the matching *Cf* gene. It was also impossible to induce HR responses after elicitor production in tomato upon PVX::PR1a*Avr4* infection, and subsequent agroinfiltration with *Cf-4*. Similarly, it was impossible to induce specific necrosis upon agroinfiltration of *Cf-4*/*Avr4*. The same experiments were repeated with *Ecp2* and candidate *Cf-Ecp2* genes, but no specific HR was induced, as expected.

Transformation of tomato with *Cf-Ecp2* candidates genes

Since agroinfiltration in *Nicotiana* species and tomato did not allow the identification of the *Cf-Ecp2* resistance gene, candidate *Cf-Ecp2* genes were tested by stable transformation to the susceptible tomato cultivar MM-Cf0. New binary constructs were made in which the candidate *Cf-Ecp2* genes are flanked by their native promoter and terminator sequences, designated as pMOG800::g*OR2* (Figure 1d). The transformation efficiency (rooting shoots per explant) was higher in the empty-vector transformation control compared to

Table 1. Data obtained from transformation experiments of tomato cultivar MoneyMaker-Cf0 with pMOG800 binary vector constructs with genomic subclones of *Cf-Ecp2* candidate genes or without any insert. Transformation efficiency indicates the number of rooting shoots per explant.

	Number of				Transformation
Binary construct	**explants**	**calli**	**shoots**	**rooting shoots**	**efficiency**
pMOG800::g*OR2A*	817	291	93	21	2.5%
pMOG800::g*OR2B*	750	313	110	38	5.1%
pMOG800::g*OR2C*	719	211	108	37	5.1%
pMOG800::-	694	257	219	69	9.9%

transformation with gOR2 inserts (Table 1). No difference in transformation efficiency was observed between transformation of hypocotyls and cotyledons (results not shown). Although the transformation efficiency in tomato was not very high, sufficient numbers of autonomous transgenic plants were generated for functional screening. Plants with full-length copies of the transgene were selected by DNA-blot hybridisation and the copy number of the transgene was determined (results not shown).

Functional screenings of tomato transgenics for candidate *Cf-Ecp2* genes

Fungal disease assays are destructive for susceptible tomato plants. Therefore, *Cf-Ecp2* mediated resistance in primary transgenic plants was investigated by assaying for Ecp2 responsiveness. First , at least 13 individual primary transformants of each candidate *Cf-Ecp2* gene were infiltrated with Ecp2 protein [10µM]. The infiltrated leaf sector of resistant Cf18 control plants (harbouring *Cf-Ecp2*) was completely necrotic at three days after infiltration, while no visible response could be observed in non-responding MM-Cf0 plants (Figure 3b). Unfortunately, plants transgenic for candidate *Cf-Ecp2* genes did not respond to the infiltrated Ecp2 protein. Recognition could be impaired by tissue culture. Therefore T_1 plants were selfed for further analysis. Twenty selfings of primary transformants (T_2 families) were analysed by infiltration with a high Ecp2 protein concentration [30µM]. Unfortunately, plants transgenic for candidate *Cf-Ecp2* genes did not respond to the infiltrated Ecp2 protein. Hence, by Ecp2 protein infiltration we could not show which of the candidate genes mediates Ecp2 recognition.

For the identification and mapping of *Cf-Ecp2* resistance, Ecp2 has been delivered into the apoplast by PVX (Laugé *et al.*, 1998a; Haanstra *et al.*, 1999). Therefore, as alternative for Ecp2 protein infiltration, PVX::PR1a*Ecp2* inoculations were performed on cuttings of primary transgenic plants to systemically overexpress the Ecp2 elicitor protein. Ten days post PVX inoculation, Cf18 plants were completely necrotic as a result of the

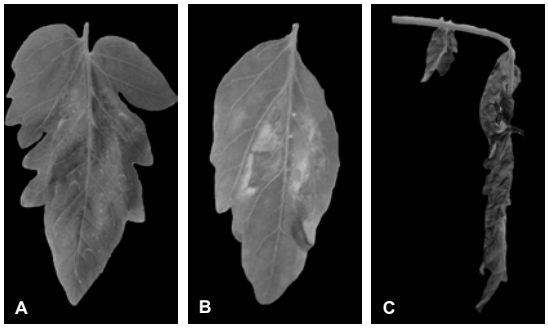

Figure 3. Comparison of responses of leaflets of *Cf-Ecp2* plants by different modes of Ecp2 delivery. (**A**) Agroinfiltration with 35S-driven *Ecp2* *Agrobacterium* culture. Only severe background chlorosis induced by *Agrobacterium* is visible. Transient expression of Ecp2 is absent. Photograph was taken 7 days post infiltration. (**B**) Specific hypersensitive response upon infiltration of 10µM Ecp2 protein. Photograph was taken 5 days post infiltration. (**C**) Specific necrosis upon PVX::PR1a*Ecp2* inoculation. Systemic necrosis was observed in *Cf-Ecp2* plants. Photograph was taken 14 days post PVX inoculation.

systemic recognition of Ecp2 (Figure 3c), whereas MM-Cf0 plants and all cuttings transgenic for the candidate *Cf-Ecp2* genes again did not show HR but only displayed mosaic symptoms as result of PVX infection.

By using the RGA-fingerprinting method (De Kock *et al*, CHAPTER 3) on individual primary transformants, transcripts of all three *Cf-Ecp2* candidate genes appeared to be absent while transcripts of other *Hcr9*s, like *NL0C*, could be identified, (Figure 2c). Consequently, the absence of transcriptional activity of candidate *Cf-Ecp2* genes directly determined the absence of Ecp2 responsiveness.

DISCUSSION

Functional complementation by transient assays in *Nicotiana* spp.

Agroinfiltration in *Nicotiana* species is a widely used method to the study functionality of *R*-genes derived from species of Solanaceae. However, we were not able by using this method to identify the functional *Cf-Ecp2* gene. DNA-instability of inserts was observed in *Agrobacterium* that resulted in recombination of the insert. This artefact is probably caused by the presence of duplicated identical sequences in the insert. Although the pCLD04541 binary cosmid vector was used for the cloning of many resistance genes, e.g. *Cf-2*, *Cf-4*, *Cf-5* and *Hero* (Dixon *et al.,* 1996; Thomas *et al.,* 1997; Dixon *et al.,* 1998, and Ernst *et al.,* 2002), this vector was apparently not suited to clone *Cf-Ecp2*. To our knowledge, DNA-instability in this vector has not been reported in literature before.

The necrotic responses in tobacco induced upon elicitor recognition by transient expression of *Cf* genes driven by their native promoter is comparable to *Cf* genes overexpressed by the 35S promoter (R. van der Hoorn and M. Kruijt, *pers. comm.*). However, it could not be excluded that the transcription level of the candidate *Cf-Ecp2* genes on the genomic clones was too low as a result of the non-functionality of the tomato

promoter regions in *Nicotiana* species. Furthermore, additional components may be required for Ecp2-induced HR as was also observed for the tomato-*C. fulvum* gene-pair *Cf-2*/*Avr2* that required *Rcr3* (Dixon *et al*., 2000), a secreted papain-like cystein endoprotease (Krüger *et al*., 2002).

Besides studies on *Cf/Avr* gene pairs (Van der Hoorn *et al.,* 2000), agroinfiltration in *Nicotiana* species has also been used to study the function of *Pto* (reviewed by Pedley and Martin, 2003; Jamir, *et al*., 2004), *Bs4* from tomato (Schornack *et al.*, 2004), and *Bs2* from pepper (Tai *et al.*, 1999). As the signal transduction pathway components to initiate HR are present in tomato, pepper, and *Nicotiana* species, and certain *Nicotiana* species recognise Ecp2 upon infiltration (CHAPTER 5), we expected that the tested *Nicotiana* species should also support *Cf-Ecp2/Ecp2* induced responses. We conclude that *Nicotiana* species are not suitable for the identification of the *Cf-Ecp2* gene, though we do not understand why.

Functional complementation by transient assays in tomato

Van der Hoorn *et al.* (2000) reported severe aspecific chlorosis and necrosis upon agroinfiltration of *Cf/Avr* gene pairs in tomato. We tried to optimise agroinfiltration in tomato to study *Cf-4/Avr4* interaction. Although agroinfiltration in tomato has previously been used to study the interaction of different gene-for-gene pairs, like the *Pto*-*Prf*/*AvrPto* (Rathjen *et al.*,1999), *R3*/*AvrPphB* (Tampakaki *et al*., 2002) and *EIX*/*LeEix* (Ron and Avni, 2004), we were not able to use agroinfiltration to study *Cf* mediated recognition of Avr4. We finally conclude that for agroinfiltration in tomato the transformation efficiency or transient expression of the transgene is insufficient to induce an unambiguous response.

Functional screening of tomato transformants

Both the complementation assays in *Nicotiana* species and tomato did not result in the identification of a functional *Cf-Ecp2*. Therefore, we continued with classical complementation by stable transformation of susceptible tomato plants with *Cf-Ecp2* candidate genes. Tomato transformation with 35S driven *Cf-4* and *Cf-9* has never been successful (B. Wulff and J. Jones, *pers. comm*). Apparently, overexpression of these *Cf* genes during tissue culture is lethal. As none of the binary cosmids or pBIV2 clones contain a single candidate *Cf-Ecp2* gene, new constructs with genomic subclones were made, each carrying only one candidate *Cf-Ecp2* gene.

Both by Ecp2 protein infiltration and Ecp2 overexpression upon PVX-inoculation, we were not able to identify the functional *Cf-Ecp2* gene in primary transformants and their progeny. Absence of transcripts of all three *Cf-Ecp2* candidate genes was shown by using the RGA-fingerprinting method (De Kock *et al*, CHAPTER 3). Transcription of transgenes in plants derived from tissue culture could be reset to normal expression levels after self-fertilisation. However, the levels of expression of the candidate *Cf-Ecp2* genes in T_2

families is not yet investigated. T_2 plants resistant to kanamycin will be used for further crossings and subsequent analyses.

***Cf-Ecp2*, a gene too far...**

Despite applying all commonly used strategies for functionality tests of *R* genes, we were not able to identify the functional *Cf-Ecp2* candidate gene. Both the map-based cloning results (De Kock *et al*., CHAPTER 2) and the *Hcr9* RGA-fingerprint results (De Kock *et al*., CHAPTER 3) showed that the *Cf-Ecp2 OR* cluster contains no more than three *Hcr9*s. Based on our present knowledge of the tomato-*C. fulvum* interaction, we still assume that one of these *Hcr9*s mediates Ecp2 recognition. By virus-induced gene silencing in tomato (Liu *et al.*, 2002) it became possible to successfully silence *Cf-4* as *C. fulvum* isolates expressing *Avr4* became virulent on *Cf-4*-silenced MM-Cf4 plants (S. Gabriels, *pers. comm.*). Application of this method on Cf18 plants using an *OR Hcr9*-specific silencing-vector may finally prove whether one of the three *OR Hcr9*s indeed is involved in Ecp2 recognition.

Expression of the candidate *Cf-Ecp2* genes in primary transgenic plants was absent or below the detection level. Promoter regions for both *OR2A*- and *OR2B*- constructs included an 1.7kb upstream promoter sequence and for the *OR2C*-construct an 2.3 kb promoter sequence was included (Figure 1d). The predicted transcription-initiation site (TATA-box) was close to the start-codon of the *Hcr9*s. These upstream sequences are expected to be sufficient for normal gene expression. The *Cf-Ecp2 OR* cluster harbours a large duplication of 2.6 kb (indicated at Figure 1a) and the first 576 bp of this duplicated region shows high sequence homology (92%) with the upstream region of *Hero*, a NBS-LRR gene located on Chromosome 4 conferring broad spectrum resistance against potato cyst nematodes (Ernst *et al.,* 2002). At both the *Hero* locus and the *OR* locus, this box is located 1.7-2.2 kb upstream of the ORF and could act as cis-acting binding domain essential for the transcriptional activity of the downstream gene. This box was also included in complementation analysis for *Hero*. However, as a result of the subcloning strategy, we were not able to completely include this region in the genomic *OR2A*- and *OR2B* binary constructs.

If the promoter sequence is insufficient in the stable transformants, it would have been expected that agroinfiltration with 35S-driven candidate *Cf-Ecp2* genes in *Nicotiana* would have been positive. Since also all 35S-driven agroinfiltration attempts failed to reveal the functional *Cf-Ecp2* gene, it is expected that at least one additional tomato component is required for the *Cf-Ecp2*/*Ecp2* mediated HR. Interestingly, a decreased necrotic response was observed in progenies of a backcross program of the *Cf-Ecp2* locus of Cf18 into MM-Cf0 (B. Brandwagt, *pers. comm.*). This suggests the presence of an HR-stimulating locus in Cf18 plants that is unlinked to *Cf-Ecp2* and is absent in MM-Cf0.

However, this quantitative nature of Ecp2 recognition was probably not observed in the genetic analysis of *Cf-Ecp2* resistance since plants were screened by overexpression of Ecp2 upon PVX inoculation (Haanstra *et al.*, 1999). A reduced HR might have been observed when the segregating population would have been analysed by *C. fulvum* disease assays.

Final remark

Cloning of the *Cf-Ecp2* gene started as a simple, step-by-step cloning procedure. We were able to isolate the *Hcr9* cluster which should be involved in *Cf-Ecp2*-mediated resistance, however, we could not identify the *OR Hcr9* that mediates Ecp2 recognition in the complementation assays. Based on all results we have to conclude that recognition of the *C. fulvum* Ecp2 elicitor is not solely mediated by one *OR-Hcr9* alone. A tomato-derived HR-stimulating factor may be required for *Cf-Ecp2*/*Ecp2*-mediated resistance. Future research has to unravel the unknown details that hampered the identification of a functional *Cf-Ecp2*.

EXPERIMENTAL PROCEDURES

Preparation of plasmid constructs for transient Agrobacterium-mediated expression

All DNA manipulations were performed using standard protocols (Sambrook *et al*., 1989). Selected *Cf-Ecp2* genomic library clones were derived from De Kock *et al.*, CHAPTER 2. Genomic agroinfiltration constructs were made by subcloning of the *Not*I inserts of pBluestar clones into the binary pBIVM2 vector [a pCGN1548 derivative (McBride and Summerfelt, 1990)].

For the construction of overexpression agroinfiltration constructs, the gene-candidates *OR2*A, *OR2*B, and *OR2*C of the *OR* sequence contig were amplified using primers-sets located at the ATG-position (OR2ABf2: 5'-ggatccatgggttacgtaaaacttgtttttttaatg-'3, OR2Cf: 5'-ggatccatgggctacgtagaccttgtat-tttttatg-3') and located at the 3' utr of the candidate genes (OR2Ar: 5'-ggcgcgcctaatgcttttattacttaggg-aaatgcac-'3; OR2Br: 5'-ggcgcgccatagagattaagttgaatacctggagg-3'; OR2Cr: 5'-ggcgcgccgaaaaatatca-agttgaatacctggag-3') with *Pfu-Turbo* DNA polymerase (Stratagene) at T_m=50°C, 30 cycli, using selected library clones as template (*Bam*HI and *Asc*I site, respectively, are underlined). PCR products were subcloned into the PCR-Script vector (Stratagene) and transformed to *E.coli* DH5α. The authenticity of cloned gene-candidates was checked by *Hcr9* fingerprinting (De Kock *et al*., CHAPTER 3). *Bam*HI/*Asc*I digested insert DNA was isolated from agarose and ligated into the binary plasmid pMK40, a pMOG800 (Honée *et al*., 1998) derived binary expression vector containing the *Nco*I/*Pst*I inserted multiple cloning site (*Nco*I-*Bam*HI-*Asc*I-*Pst*I), modified from pRH80 (Van der Hoorn *et al*., 2000) flanked by the CaMV 35S promoter and potato proteinase inhibitor-II (PI-II) polyadenylation cassette. The resulting pMOG800::35S*OR2* expression constructs were named according to the gene present in the T-DNA. For transient Ecp2 production, the PR1a-Ecp2 ORF from the PVX::*Ecp2* construct (Laugé *et al.,* 1998a) was subcloned into the multiple cloning site of pRH80 flanked by the 35S promoter and the PI-II terminator. Subsequently, the promoter-ORF-terminator cassette was cloned into pMOG800, creating the binary plasmid pEcp2.

All binary constructs were transformed by electroporation to *Agrobacterium tumefaciens* strain GV3101. The integrity of the plasmids and cosmids was tested by restriction analysis of plasmids or cosmids isolated from *Agrobacterium* cultures used for agroinfiltration.

Transient Agrobacterium-mediated expression

Transient Agrobacterium-mediated expression (agroinfiltration) of *Nicotiana* species (*N. bethamiana* TW16*, N. glutunosa* TW58*, N. kawakamii* TW72, and *N. tomentosiformis* TW142) and tomato MoneyMaker-Cf0 (MM), MM-Cf4, and Cf18 (harboring the *Cf-Ecp2* gene), was essentially performed as described by Van der Hoorn *et al.* (2000) or Rathjen *et al.* (1999). The binary expression constructs p*Cf-4*, p*Avr*4, p*Cf-9*, and p*Avr9* (Van der Hoorn *et al.,* 2000) were used as control gene-pairs during agroinfiltration assays. Cultures expressing an *Hcr9* or containing genomic inserts were mixed in a 1:1 ratio with a culture expressing the *Avr* or *Ecp*2 gene and infiltrated in expanded *Nicotiana* or tomato leaves. In infiltration series in which more than two different expression constructs or apoplastic washing fluid were combined, cultures or samples were mixed in equal ratio's. Apoplastic washing fluids of Cf18 plants, non-treated and *C. fulvum* race 5.kim diseased MM-Cf0 plants were prepared according to the method described by De Wit and Spikman (1982). For the systemic production of Ecp2, plants were inoculated with PVX::PR1a*Ecp*2 according to Laugé *et al*. (1999) one week (for *Nicotiana* spp.) or two weeks (for tomato) before agroinfiltration.

Preparation of plasmid constructs for plant transformation

For the construction of genomic subclones, *Cf-Ecp2* candidate genes flanked by their native promoter and terminator region were subcloned into a binary vector (Figure 1d). Cosmid DNA of clone 3.8G encoding *OR2A* was *Eco*RI/*Bam*HI digested, plasmid DNA of pBlueStar clone 49 harboring *OR2B* was digested with *Eco*RI and plasmid DNA of pBlueStar clone J containing *OR2C* was *Xho*I/*Bam*HI digested. Digestion mixtures were size-separated on a 0.7% agarose gel and specific bands were isolated from agarose gel (Zymo Research), ligated in (with matching restriction enzymes) digested and dephosphorylated pMOG800 vector and transformed to *E. coli* DH5α. The resulting pMOG800::g*OR2* constructs were named after the gene present on the T-DNA. By *Hcr9* fingerprinting (De Kock *et al*, CHAPTER 3), clones containing a specific *Cf-Ecp2* gene-candidate were identified and by restriction analysis, the correct insert size was investigated. Binary constructs containing the appropriate genomic subclones were subsequently transferred by electroporation to *A. tumefaciens* LBA4404. The integrity of the binary plasmids was tested by restriction analysis isolated plasmids isolated from *Agrobacterium* cultures used for transformation.

Generation of tomato transformants

Plant transformation was essentially performed as described by Ling *et al.* (1998). Both cotelydons and hypocotyls of the *L. esculentum* cultivar MM-Cf0 were used for transformation. Preculture of explants on a tobacco cell suspension was omitted. Basic culture medium containing Murashige-Skoog salts with vitamins, 1% (w/v) sucrose, 1% (w/v) glucose, 0.8% (w/v) agar, pH 5.8, was supplemented with 2 mg/l zeatin, 0.02 mg/l IAA, 200 mg/l timetin, 100 mg/l kanamycin, and 200 mg/l vancomycin for callus induction. Zeatin concentration was reduced to 1 mg/l for shoot-induction. Root-inducing medium was supplemented with 0.02 mg/l IAA, 200 mg/l timetin, and 50 mg/l Kanamycin. Rooting primary transformants were transplanted to soil for further analysis and propagation. Genomic DNA of primary transformants was isolated according to Van der Beek *et al.* (1992). DNA-blot hybridisation experiments were performed to analyse complete integration of the insert and the copy number of the insert. Primary transformants (T_1) with single and double integration of the complete transgene were selected self-fertilisation, creating T_2. Theoretically, 25 percent of the single-

copy T_2 plants does not contain the transgene. These plants were selected by kanamycin-spray according to Weide *et al*. (1989) and removed before self-fertilisation of single copy T_2 plants (generating T_2S_1).

Functional screening of tomato transformants

An (N-terminal HIS-FLAG-tagged) Ecp2 protein was produced in the *Pichia pastoris* expression system (Invitrogen) and affinity purified (Brandwagt, *in preparation*). Protein-dilution series were infiltrated in fully expanded leaves. Responses induced by Ecp2-recognition were scored three to seven days after infiltration. Cuttings were made to perform destructive PVX::PR1a*Ecp*2 disease tests as descibed by Laugé *et al.* (1998a). Responses induced by PVX::PR1a*Ecp*2 were scored seven to fourteen days after PVX inoculation.

Hcr9 mRNA profiling by RGA-fingerprinting

Total RNA was isolated from susceptible MM-Cf0, resistant tomato Cf18 plants (harbouring the *Cf-Ecp2* resistance gene) and individual primary transformant plants carrying a single copy *Cf-Ecp2* candidate gene with Trizol Reagent (Life Technologies) from approximately 0.2 g fresh leaf material according to suppliers protocol. RNA was DNaseI treated and purified using Gene-elute total RNA purification columns (Sigma). Ten µg total RNA was used for first-strand cDNA synthesis. The *Hcr9* resistance gene analogue fingerprinting method was applied on first-strand cDNA as described in De Kock *et al*., CHAPTER 3.

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Chapter 5

Recognition of *Cladosporium fulvum* Ecp2 elicitor by non-host *Nicotiana spp.* is mediated by a single dominant gene that is not homologous to known *Cf*-genes

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ABSTRACT

Cladosporium fulvum is a fungal pathogen of tomato that grows exclusively in the intercellular spaces of leaves. Ecp2 is one of the elicitor proteins that is secreted by *C. fulvum* and is specifically recognised by tomato plants containing the resistance gene *Cf-Ecp2*. Recognition is followed by a hypersensitive response (HR) resulting in resistance. HR-associated recognition of Ecp2 has been observed in *Nicotiana paniculata*, *N. sylvestris*, *N. tabacum* and *N. undulata* that are non-host plants of *C. fulvum*. Absence of Ecp2-recognition did not lead to growth of *C. fulvum* on *Nicotiana* plants. We show that HR-associated recognition of Ecp2 is mediated by a single dominant gene in *N. paniculata*. However, based on PCR- and hybridisation analysis this gene is not homologous to known *Cf*-genes.

INTRODUCTION

Cladosporium fulvum is a biotrophic fungus that causes leaf mold of tomato. *C. fulvum* enters tomato leaves through stomata and obtains nutrients via enlarged intercellular hyphae that are in close contact with the host mesophyll cells. No specialised feeding structures, such as haustoria, are formed during infection. About two weeks after penetration, when intercellular spaces are fully colonised, conidiophores emerge from stomata and numerous conidia are released that can cause secondary infections. During colonisation, *C. fulvum* secretes at least eight known avirulence (Avr) proteins and 'extracellular proteins' (Ecp) into the apoplastic space (Joosten and De Wit, 1999). Four are race-specific (Avr2, Avr4, Avr4E and Avr9) whereas for the others (Ecp1, Ecp2, Ecp4 and Ecp5) race-specificity has not yet been observed. All Avr proteins trigger hypersensitive response (HR)-associated defence responses in tomato plants with the matching *Cf* resistance genes (Joosten and De Wit, 1999). Furthermore, individual accessions within the *Lycopersicon* genus have been identified that react with an HR to each of the individual Ecp proteins. Matching *R*-genes, designated *Cf-Ecps*, have been introgressed into commercially grown tomato (*L. esculentum*) for further analysis (Laugé *et al.*, 1998a; Haanstra *et al.*, 2000; Laugé *et al.*, 2000). *Cf-Ecp2*, *Cf-Ecp3* and *Cf-Ecp5* may be homologues of the *Cf-9* resistance gene (*Hcr9s)* that encodes an extracellular plasma membrane-anchored leucine-rich repeat (LRR) receptor-like protein (Haanstra *et al.*, 1999; Yuan *et al.*, 2002).

The host range of *C. fulvum* is restricted to the genus *Lycopersicon* (Bond, 1938). This limits the identification of HR-based activity of *C. fulvum* proteins in other plant species by

inoculation with *C. fulvum* isolates. However, HR-associated recognition of Avr factors in non-host plants of *C. fulvum* was tested by using PVX-mediated expression of the elicitor genes. Laugé *et al.*, (2000) showed that two of the three accessions of *Nicotiana paniculata* respond with an HR to Ecp2. In *Lycopersicon* species PVX-mediated expression of an *Avr* or *Ecp* gene leads to systemic HR-associated recognition (Hammond-Kosack *et al.*, 1995; Joosten *et al.*, 1997, Luderer *et al.*, 2002a,b), but in *N. paniculata*, plants respond with a local HR to PVX-mediated expression of *Ecp2* (Laugé *et al.*, 2000).

Little is known of the genetic basis of non-host resistance and the perception of elicitor proteins. Nonhost resistance can occur without any visible symptoms whereas another types of nonhost resistance results in a rapid HR (reviewed by Mysore and Ryu, 2004). Collins *et al*, (2003) showed that the initiated resistance mechanisms upon penetration of *Blumeria graminis* f.sp. *hordei* in *Arabidopsis* are dispensable from immunity mediated by race-specific resistance genes. In contrast, Vleeshouwers *et al.*, (2000) and Huitema *et al.*, (2003) revealed a significant overlap in HR-associated nonhost resistance responses to *Phytophthora infestans* with gene-for-gene resistance responses. However, it is unknown whether non-host based HR is functionally related to resistance or whether it is a consequence of spontaneous evolution of *R*-genes in non-host plants which frequently results in recognition of non-self proteins. Here, we provide genetic, cytological and molecular data on Ecp2-recognition in *Nicotiana* species that are non-hosts for *C. fulvum*. We show that recognition of Ecp2 in *N. paniculata* is governed by a single dominant gene that is likely not an *Hcr9*. The possible role of Ecp2-recognition by non-host plants is being discussed.

RESULTS

Specific HR-associated recognition of Ecp2 by several non-host species

To extend our study of the non-host recognition of Ecp2, 71 accessions of 38 *Nicotiana* species, including three additional accessions of *N. paniculata* were tested for recognition of Ecp2 (Table 1). Upon PVX::PR1a*Ecp*2 inoculation, the Ecp2 protein is secreted to the apoplastic space due to the presence of the pathogenesis-related protein 1a (PR1a) signal sequence. Most of the PVX::PR1a*Ecp*2-inoculated *Nicotiana* plants showed only mosaic symptoms, indicating that the plants are susceptible to PVX and do not respond to Ecp2. Specific HR-associated recognition of Ecp2 was found in some of the newly tested *N. paniculata* accessions. In addition, all tested *N. sylvestris, N. tabacum* and *N. undulata* accessions responded with HR to Ecp2. HR-associated recognition of elicitor proteins produced by the PVX-vector in *Lycopersicon* species develops systemically and

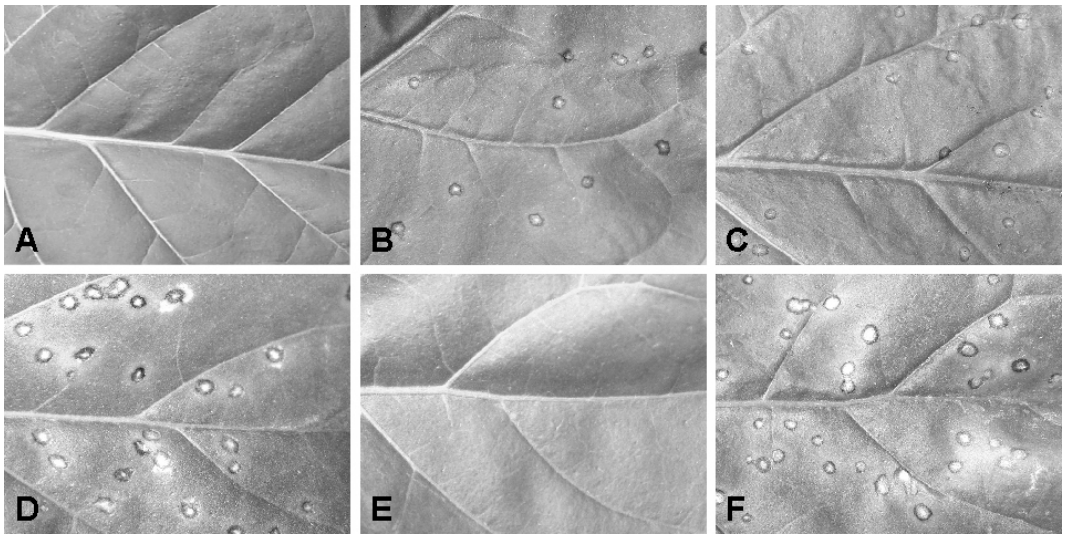

Figure 1. Responses induced by PVX::PR1a*Ecp*2 on leaves of *Nicotiana* species seven days post inoculation. No response was observed in *Nicotiana tomentosiformis* (**A**) and *N. paniculata* accession TW100 (**E**), whereas local necrotic lesions (hypersensitive response) are visible on leaves of *N. tabacum* (**B**), *N. sylvestris* (**C**) and *N. paniculata* TW99 (**D**) and TW101 (**F**).

becomesvisible nine days post PVX::PR1a*Ecp*2 inoculation. In *Nicotiana* plants, however, HR-associated recognition of Ecp2 is detectable four days post PVX::PR1a*Ecp*2 inoculation and remains confined to the inoculated leaves (Figure 1). This indicates that Ecp2-induced HR arrests the systemic spread of PVX::PR1a*Ecp*2 and suggests that PVX expressing *Ecp*2 has become avirulent on these *Nicotiana* plants. Some of the tested accessions appeared to be naturally resistant to wt-PVX as there was no systemic spread of wt-PVX (Table 1). Our results confirmed earlier results reported by Van Dijk and Cuperus (1989) who found these accessions to be resistant to PVX.

In Solanaceous species, the majority of identified *R* genes encode intracellular proteins. In addition to extracellular delivery of Ecp2 by PVX, we also tested the HR-associated recognition of Ecp2 when it was delivered without a signal peptide (designated as PVX::*Ecp*2). Without a signal peptide, Ecp2 is expected to remain in the cytoplasm. In tomato, HR-associated recognition of the truncated Ecp2 protein was absent and resulted in systemic mosaic symptoms (Table 2). In contrast, Ecp2-responding *Nicotiana* species did react with an HR upon inoculation with PVX::*Ecp*2, but the response was one- to two days delayed compared to inoculation with PVX::PR1a*Ecp*2. Consequently, PVX-derivatives could escape from the less efficient arrest by PVX::*Ecp*2-induced HR which resulted in little systemic spread and some systemic HR.

To get a better understanding of Ecp2-recognition by non-host plants, the HR response was studied by Ecp2 production through agroinfiltration or by manual infiltration of a purified Ecp2 protein. All plants responding with an HR upon PVX::PR1a*Ecp*2 inoculation, also responded with an HR upon Ecp2 production through agroinfiltration or by manual

Table 1. Hypersensitive responses induced in various *Nicotiana* species upon inoculation with PVX::PR1a*Ecp*2.

Subgenus/ Section	*Species*	Plant identification numer	Designation seed repository	Response [a] to PVX::PR1a*Ecp*2
Petunioides				
Acuminatae	*N. acuminata*	PI 555469	TW04	-
	N. acuminata	PI 42347	TW05	-
	N. attenuata	PI 555476	TW13	-
	N. corymbosa	PI 114824	TW35	-
	N. linearis	PI 555530	TW77	-
	N. miersii	PI 555537	TW85	-
	N. spegazzinii	PI 281756	TW124	-
Alatae	*N. bonariensis*	PI 555489	TW28	-
	N. forgetiana	PI 555501	TW50	-
	N. langsdorffii	PI 42337	TW74	-
	N. longiflora	PI 555533	TW80	-
	N. plumbaginafolia	PI 555548	TW106	-
	N. plumbaginafolia	PI 302476	TW107	-
	N. plumbaginafolia	PI 302478	TW108	-
	N. sylvestris	PI 555569	TW136	HR
	N. sylvestris	PI 555570	TW137	HR
	N. sylvestris	PI 555571	TW138	HR
Bigelovianae	*N. clevelandii*	PI 555491	TW30	-
Noctiflorae	*N. acaulis*	PI 555468	TW01	-
	N. petunioides	PI 555547	TW105	-
Nudicaules	*N. nudicaulis*	PI 555540	TW90	-
Suaveolentes	*N. bethamiana*	PI 555478	TW16	-
	N. gossei	PI 230953	TW68	-
	N. maritima	PI 555535	TW82	-
	N. occidentalis	PI 555541	TW91	-
Undulatae	*N. undulata*	PI306637	TW147	HR
Rustica				
Paniculatae	*N. benavidesii*	PI 555477	TW15	R
	N. cordifolia	PI 555493	TW33	-
	N. cordifolia	PI 555494	TW34	-
	N. glauca	PI 555504	TW53	-
	N. glauca	PI 282690	TW54	R
	N. glauca	PI 307908	TW55	R
	N. glauca	PI 407404	TW56	-

Table 1. *continued*

Subgenus/ Section	*Species*	Plant identification numer	Designation seed repository	Response [a] to PVX::PR1a*Ecp2*
Rustica				
Paniculatae	*N. glauca*	PI 555686	TW57	-
	N. knightiana	PI 555527	TW73	-
	N. paniculata	NSL 75784	40A-G	-
	N. paniculata	NSL 75785	40B-G	HR
	N. paniculata	PI 241769	TW100	-
	N. paniculata	PI 266380	TW101	HR
	N. paniculata	PI 555544	TW103	HR
	N. paniculata	PI 555545	TW99	HR
	N. pauciflora	PI 555546	TW104	-
	N. petunioides	PI 555547	TW105	-
	N. raimondii	PI 555550	TW102	-
	N. raimondii	PI 555549	TW109	-
	N. solanifolia	PI 555558	TW123	-
Rusticae	*N. rustica*	PI 555554	TW117	-
	N. rustica	PI 555555	TW118	-
	N. rustica	PI 555692	TW119	-
	N. rustica	PI 555693	TW120	-
Thyrsiflorae	*N. thyrsiflora*	NSL 8697		-
Tabacum				
Genuinae	*N. tabacum*	NIJ874750046		HR
	N. tabacum cv. Havana	NIJ914750044		HR
	N. tabacum cv. Petit Havana	NIJ904750309		HR
	N. tabacum cv. Petit Havana (SR1)	NIJ904750317		HR
	N. tabacum cv. Samsun NN	NIJ904750310		HR
	N. tabacum cv. White Burley	NIJ984750038		HR
Tomentosae	*N. glutinosa*	PI 555507	TW58	-
	N. glutinosa	PI 555510	TW59	-
	N. glutinosa	PI 241768	TW60	-
	N. glutinosa	PI 555505	TW61	-
	N. glutinosa	PI 555508	TW63	-
	N. glutinosa	PI 555509	TW64	-
	N. glutinosa	PI 555511	TW65	-
	N. glutinosa	PI 555512	TW66	-
	N. kawakamii	PI 459106	TW72	-
	N. othophora	PI 235553	TW95	-
	N. setchellii	PI 555557	TW121	-
	N. tomentosa	TW 141	TW141	R
	N. tomentosiformis	PI 555572	TW142	-

Table 2. Responses of tomato or *Nicotiana* species induced by different proteins delivered by PVX, agroinfiltration or manual protein infiltration.

Species	PVX::PR1a*Ecp2*[a]	PVX::*Ecp2*[a]	Agroinfiltration				Ecp2 protein
			Ecp2	***Avr4***	***Cf-4***	***Cf-4/Avr4***	**[15µM]**
tomato MM-Cf0	mosaic [b]	mosaic	n.t.	n.t.	n.t.	n.t.	-
tomato Cf-Ecp2	HR [c]	mosaic	n.t	n.t	n.t	n.t	HR
N. sylvestris	HR	HR	HR	- [e]	-	HR	HR
N. tabacum	HR	HR	HR	-	-	HR	HR
N. undulata	HR	n.t. [d]	HR	-	-	HR	-
N. paniculata TW99	HR	HR	HR	-	-	HR	chlorosis
N. paniculata TW100	mosaic	mosaic	-	-	-	HR	-
N. paniculata TW101	HR	HR	HR	-	-	HR	HR

(a) PVX::PR1a*Ecp*2 derivative with PR1a signal peptide that allows secretion of Ecp2, or PVX::*Ecp*2 derivative without signal peptide preventing secretion of Ecp2.
(b) lack of Ecp2-recognition resulting in mosaic symptoms caused by systemic PVX spread.
(c) Ecp2-recognition resulting in a hypersensitive response at the inoculated leaf or infiltrated region.
(d) not tested.
(e) no response.

(a) -: mosaic symptoms (no response to Ecp2); HR: local hypersensitive response; R: natural resistance to PVX;

infiltration of a purified Ecp2 protein. All plants responding with an HR upon PVX::PR1a*Ecp*2 inoculation, also responded with an HR upon Ecp2 production through agroinfiltration (Table 2). None of the plants responded with HR upon separate delivery of either Cf-4 or Avr4 by agroinfiltration. In all plants, co-agroinfiltration of the matching *Cf-4*/*Avr4* gene-pairs resulted in an HR. All plants, including *N. paniculata* TW100 that does not respond to Ecp2 responded with an HR upon co-agroinfiltration of *Cf-4/Avr4*, indicating that the non-responsiveness is not due to ineffective agroinfiltration.

Infiltration of leaves with Ecp2 protein resulted in a specific HR in a tomato line harbouring *Cf-Ecp2* (Table 2). HR-associated recognition of Ecp2 was also observed in *N. sylvestris*, *N. tabacum* and *N. paniculata* TW101. In *N. paniculata* TW99 the response to Ecp2 protein infiltration was weaker showing chlorosis associated with little necrosis. *N. undulata* did not respond with an HR upon Ecp2 protein infiltration (Table 2). Addition of 0.2% Tween-80 to Ecp2 may promote the uptake of extracellular proteins (Brandwagt *et al.*, 2001b) but this addition did not significantly increase the HR response in *N.undulata* and *N. paniculata* TW99.

Lack of Ecp2-recognition does not allow growth of *C. fulvum* on non-host plants

The host range of *C. fulvum* is restricted to the genus *Lycopersicon* and *Nicotiana* species are non-hosts of *C. fulvum* (Bond, 1938). It was investigated whether Ecp2-recognition by some *Nicotiana* species is one of the causes of this non-host resistance. *N. tabacum*, *N. paniculata* accessions TW99, TW100 and TW101 and tomato plants either responding or not responding to Ecp2 were inoculated with an Ecp2 producing transgenic *C. fulvum* strain also expressing β-glucuronidase (GUS).

In tomato lacking *Cf-Ecp2,* successful colonisation of the apoplast was observed (Figure 2a), resulting in sporulation 18 days after inoculation. In Cf-Ecp2 tomato plants, the fungus penetrated leaves via the stomata, but intercellular colonisation was arrested due to recognition of Ecp2 resulting in an HR response. On the non-host plants *N. tabacum* and *N. paniculata*, the conidia of *C. fulvum* germinated and produced runner hyphae, but growth of the intercellular hyphae was arrested already during penetration of stomata (Figures 2a and 2b). In rare cases, penetration of stomata did occur, but further growth was soon arrested. No difference in penetration efficiency was observed between *N. paniculata* accession TW99 and TW101 (which both respond to Ecp2) and TW100 (which does not respond to Ecp2). Lack of Ecp2-recognition in TW100 did not increase penetration efficiency and colonisation by *C. fulvum*. Thus, for the arrest of fungal growth in non-host plants Ecp2-induced HR is not crucial. Other host factors or lack of essential virulence factors by *C. fulvum* to colonise *Nicotiana* species may be the cause of non-host resistance.

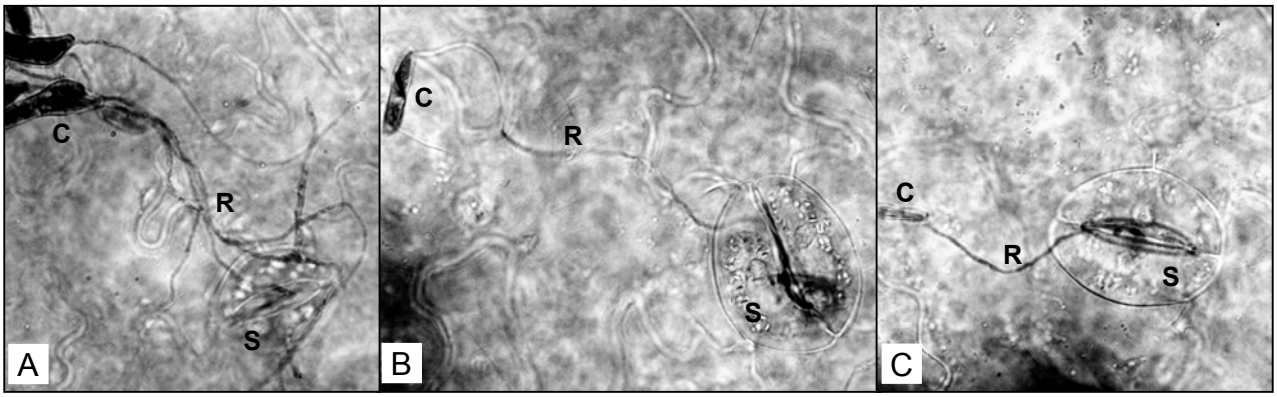

Figure 2. Colonisation of leaves by an Ecp2 producing strain of *Cladosporium fulvum* transgenic for β-glucuronidase (GUS) four days post inoculation. Germinated conidiospores (C) and runner hyphae (R) on the surface of the abaxial side of a leaf. In tomato MM-Cf0 (**A**) the runner hyphae penetrated the stomata (S) and intercellular hyphae subsequently colonise the apoplastic space at later stages. (**B**) In both *Nicotiana paniculata* TW99 (which responds with HR to Ecp2) and (**C**) *N. paniculata* TW100 (which does not respond with HR to Ecp2) the conidiospores germinate and produce runner hyphae. At the time of penetration of the stomata, fungal growth is arrested and colonisation of the apoplast is absent.

Table 3. Inheritance of HR-associated recognition of Ecp2 in *Nicotiana paniculata* accessions and their progeny.

N. paniculata **accession / progeny**	**Response to PVX inoculation** [a]		χ^2 **value**
	HR	**mosaic**	
P			
TW099	12	0	
TW100	0	12	
TW101	12	0	
F_1			
TW099 x TW100	12	0	
TW101 x TW100	12	0	
F_2			E = 3:1
TW099 x TW100	27	9	0.000 (*P* = 1.00)
TW101 x TW100	16	6	0.061 (*P* = 0.81)
BC_1			E = 1:1
(TW099 x TW100) x TW100	50	39	1.360 (*P* = 0.24)
(TW101 x TW100) x TW100	22	24	0.087 (*P* = 0.77)
Testcross			E = 1:0
(TW099 x TW101) x TW100	50	0	-

[a)] Number of plants responding with local HR or systemic mosaic symptoms upon inoculation with PVX::PR1a*Ecp*2.

HR-associated recognition of Ecp2 is mediated by a single dominant gene

The recognition of Avr factors in host plants is usually controlled in a gene-for-gene fashion by a single dominant gene (Joosten and De Wit, 1999). To determine whether a single dominant gene also confers HR-associated recognition of Ecp2 in *Nicotiana* species, the genetic basis of Ecp2-recognition was investigated. For this genetic study *N. paniculata* was used as this is the only species for which we found Ecp2-responding and non-responding accessions. To analyse the genetic homogeneity, *N. paniculata* accessions TW99 and TW101, both responding to Ecp2 and accession TW100, not responding to Ecp2, were selfed and the progenies (S_1) were inoculated with PVX::PR1a*Ecp*2.

All selfed progeny plants of the accessions TW99 and TW101 responded with a local HR to PVX::PR1a*Ecp*2, whereas systemic mosaic symptoms were observed in the TW100 progeny plants (Table 3). These results indicate that plants of the original accessions were homozygous for Ecp2-responsiveness. Isolation of systemically spread PVX::PR1a*Ecp*2 virus from infected TW100 plants and re-inoculation of this virus onto TW99, TW101 and tomato *Cf-Ecp2* plants resulted in again HR, whereas inoculation onto control tomato MM-Cf0 plants resulted in systemic mosaic symptoms. These results

Table 4. Sizes of PCR-amplification products of domains of resistance genes derived from different plant species

***R*-gene, domain**[a]	**Sizes of PCR-amplification products (kb)** [b]					
				Nicotiana paniculata		
	Tomato	**Potato**	***N. tabacum***	**TW99**	**TW100**	**TW101**
Cf-9, A-LRR 17 [c]	1.5	1.4, 1.5	-	-	-	-
Cf-9, LRR1-17 [c]	0.95, 1.2	1.1, 1.2	-	-	-	-
Cf-9, LRR18 - G [c]	1.2	1.2	-	-	-	-
Cf-2.1, A-LRR27 [d]	2.2	2.2	-	-	-	-
Cf-2.1, LRR27-G [d]	1.1	1.1	-	-	-	-
N, NBS-region	-	0.7	0.7	0.7	0.7	0.7
Pto, kinase domain	0.6	0.6	-	-	-	-

(a) see Experimental Procedures for further details.
(b) -: no PCR product obtained.
(c) domains are as described in Jones *et al.* (1994).
(d) domains are as described in Dixon *et al.* (1996).

indicate that PVX::PR1a*Ecp*2 is virulent on TW100 plants but avirulent on TW99 and TW101 plants.

Both $TW99_{S1}$ and $TW101_{S1}$ were crossed to $TW100_{S1}$. All F_1 plants displayed a local HR upon PVX::PR1a*Ecp*2 inoculation. Therefore, it can be concluded that recognition of Ecp2 inherits as a dominant character. Reciprocal crosses were performed but the crosses showed unilateral incompatibility resulting in early abortion of seed development when TW100 was used as pollen donor. The F_1 populations were both selfed and back-crossed to TW100 resulting in F_2 and BC_1 populations, respectively. From accession TW99, 36 F_2 and 89 BC_1 plants and from accession TW101, 22 F_2 and 44 BC_1 plants were inoculated with PVX::PR1a*Ecp*2 (Table 3). Of both F_2 populations about 75% of the plants showed a local HR, while of the BC_1 populations from TW99 and TW101 approximately 50% of the plants showed a local HR after inoculation with PVX::PR1a*Ecp*2. A testcross population [(TW99 x TW101) x TW100] was generated to analyse whether the genes responsible for the Ecp2-recognition in TW99 and TW101 are allelic (Table 3). From this allelic testcross population all 50 PVX::PR1a*Ecp*2-inoculated plants responded with an HR, indicating that Ecp2-recognition in *N. paniculata* accession TW99 and TW101 is mediated by a single dominant gene that is located at the same locus in both accessions.

No correlation between Ecp2-recognition and presence of known *R*-gene homologues.
Ecp2-recognition in tomato cosegregated with an *R*-gene locus containing *Hcr9*s (Haanstra *et al.*, 1999). In a search for genes mediating Ecp2-recognition in *Nicotiana* species, degenerate primers were used to amplify parts of *R*-gene homologues from

Table 5. Hybridisation of genomic DNA from tomato and different *Nicotiana* species with probes derived from various resistance genes.

Probe [a]	Species			
	tomato	***N. sylvestris***	***N. tabacum***	***N. paniculata***
Cf-9, 3'-region	+++ [b]	-	-	-
Cf-2, 3'- region	++	-	-	-
N, NBS-region	+	+++	+++	++
Pto, activation domain	+++	++	++	+

(a) see Experimental Procedures for further details.

(b) different intensities of hybridisation: - = no hybridisation; + = weak hybridisation; ++ = medium hybridisation; +++ = strong hybridisation. See also Figure 3 for hybridisation intensities. Blots were washed at medium stringency (1x SSC, 0.1% SDS at 65°C).

genomic DNA of *N. tabacum* and *N. paniculata* (Table 4). Tomato genomic DNA was included as a positive control. Genomic DNA of potato was included as a close relative of tomato that is a non-host for *C. fulvum* but contains *Hcr9* sequences.

PCR-amplification with primers complementary to the 5' region of *Hcr9*s, to amplify the variable LRR region and primers located in the more conserved 3' part of *Hcr9*s did not result in PCR products using *N. tabacum* and *N. paniculata* genomic DNA as template. PCR-amplification with these two primer sets using tomato and potato genomic DNA resulted in PCR products of estimated sizes (Table 4). Similarly no PCR products were detected in *Nicotiana* species using *Cf-2*- and *Pto*-derived specific primers, whereas both *Nicotiana* species and potato showed PCR products of estimated size using *N* gene-specific primers.

To get more information on the copy number of *R*-gene homologues in *Nicotiana* species, several DNA blot hybridisations were performed using PCR products obtained by different primer/template combinations as probe (Table 5). The tomato *Cf-9* probe showed distinct hybridisation patterns with tomato genomic DNA while aspecific hybridisation was observed with *Nicotiana* genomic DNA. Also hybridisation using *Cf-2*-derived probes did not result in distinct hybridisation patterns with *Nicotiana* species compared to clear hybridisation with tomato. Strong hybridisation with the *N* gene-derived probe with multiple fragments was observed with *Nicotiana* species, while tomato showed very weak hybridisation (Figure 3). The *Pto*-derived probe showed distinct hybridising fragments in all species. Strong hybridisation was observed in tomato, medium hybridisation in *N. tabacum* and *N. sylvestris* but only weak hybridisation with *N. paniculata*.

Restriction-fragment-length-polymorphism (RFLP) analysis using six different enzymes and probes corresponding to the different *R* genes was performed on the *N.*

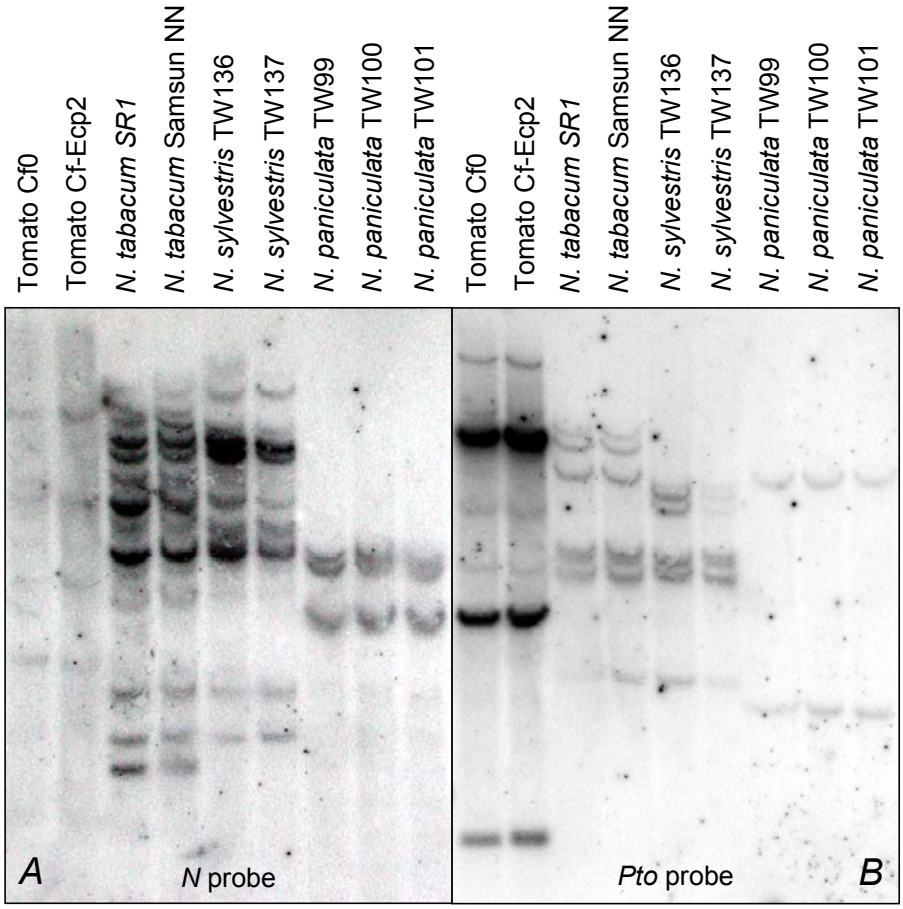

Figure 3. DNA gel blot analysis of different plant species to detect homologues of known resistance genes. **A.** Genomic DNA was digested with *Eco*RI and subsequently hybridised with a probe covering the nucleotide-binding site of the *N* gene. **B.** Genomic DNA was digested with *Bgl*II and subsequently hybridised with a probe covering the kinase domain of the *Pto* gene. 5 µg of restricted DNA was applied in each lane. Following hybridisation, blots were washed at medium stringency (1x SSC, 0.1% SDS at 65°C).

paniculata F_2 population (TW101 x TW100) that segregates for Ecp2-recognition. No hybridisation polymorphisms were observed which cosegregated with Ecp2-recognition.

DISCUSSION

HR-associated recognition of Ecp2 in non-host plants

For all Avr and Ecp proteins secreted by *C. fulvum*, corresponding *Lycopersicon* genotypes with HR-associated recognition have been identified. In *Lycopersicon*, only a limited number of accessions respond to an Avr or Ecp elicitor and they all appear to contain a matching *Cf*-gene (Laugé *et al.*, 1998a; Haanstra *et al.*, 2000; and M. Kruijt, *pers. comm.*). In addition to the *N. paniculata* accessions reported by Laugé *et al.* (2000)

we found more *Nicotiana* species responding with HR upon Ecp2-recognition. In contrast to *Lycopersicon* species, Ecp2-recognition in *Nicotiana* species is frequent and occurs in the majority of the accessions. Similarly, various members of the family of elicitin-like proteins of *P. infestans* and several novel *Phytophthora* proteins that are unrelated to elicitins, have been shown to trigger HR-like symptoms in *Nicotiana* species associated with resistance to *P. infestans* (Kamoun *et al*., 1998; Kamoun, 2001; Fellbrich *et al.*, 2002; Qutob *et al.*, 2002; Torto *et al*., 2003). In addition, non-host resistance of parsley to *Phytophthora* spp. is triggered by the recognition of a conserved 13 amino-acid sequence (Pep-13) (Nürnberger *et al.*, 1994; Nennstiel *et al.*, 1998; Brunner, *et al.*, 2002). Unfortunately, absence of genetic variation in the non-host plants responding to *Phytophthora* elicitors hampers genetic studies on the molecular basis of this recognition. Plants can also specifically respond to proteins produced by non-pathogens. For example, ethylene-inducing xylanase (EIX) from *Trichoderma viride* can elicit defence responses in species like tomato and tobacco and is controlled by a single dominant locus (Bailey *et al.*, 1993; Ron *et al.*, 2000), that codes for a *Hcr9*-like protein in tomato (Ron and Avni, 2004).

We found that a single dominant gene mediates HR-associated Ecp2-recognition in *N. paniculata* and possibly also in *N. sylvestris*, *N. tabacum* and *N. undulata*. As all accessions of the latter *Nicotiana* species responded to Ecp2, no segregating population could be generated. Since the identified *Nicotiana* species belong to different subgenera, most likely Ecp2-recognition is an ancient trait.

The tomato pathogen *C. fulvum* does not infect *Nicotiana* species (Bond, 1938) and is molecularly classified in the *Mycosphaerella* genus (Goodwin *et al*., 2001; Crous *et al.*, 2001). Members of *Mycosphaerellaceae* that are able to infect *Nicotiana* species, are *Cercospora nicotianicola*, *C. apii* (Crous and Braun, 2003) and *Septoria lycopersici* (Bouarab *et al.*, 2002). The presence of Ecp2 homologues in these fungi is unknown, but Ecp2-recognition in *N. paniculata*, *N. sylvestris*, *N. tabacum* and *N. undulata* might be a result of co-evolution between these host species and pathogens producing Ecp2-like proteins. Alternatively, Ecp2-recognition could have been generated randomly as a result of a surveillance system for the detection of non-self proteins analogous to the animal innate immunity system (Laugé *et al.*, 2000; Cohn *et al.*, 2001).

Molecular basis of Ecp2-recognition

The protein responsible for Ecp2-recognition possibly represents a guard or a virulence target of Ecp2. We searched for homologues of known *R* genes involved in Ecp2-recognition. In tomato, Ecp2-mediated resistance cosegregates with *Hcr9*s (Haanstra *et al.*, 1999, De Kock *et al*i, CHAPTER 2 and CHAPTER 3). Therefore, we investigated whether *Hcr9*s could be involved in Ecp2-recognition in *Nicotiana* species. Although several *Cf-9*-

like sequences are present in Solanaceous species (www.ncbi.nlm.nih.gov and www.tigr.org), we could not detect *Cf-9*- or *Cf-2*-like sequences in *Nicotiana* species.

In tomato the *C. fulvum* elicitors are presumed to be perceived extracellularly. However, Ecp2-recognition in *Nicotiana* species could also be cytoplasmic since intracellular production of Ecp2 resulted in HR. The absence of HR upon Ecp2 infiltration in *N. undulata* may be caused by the lack of cellular uptake. Alternatively, protein leakage from the cytoplasm into the apoplast could occur to enable extracellular recognition.

The majority of the intracellular *R* proteins contain LRR- and nucleotide-binding site (NBS) domains. The best studied NBS-LRR resistance gene from tobacco is the *N* gene, that mediates resistance towards the Tobacco Mosaic Virus (TMV) by recognition of the viral replicase protein (Whitham *et al.*, 1994, Erickson *et al.*, 1999). Another class of intracellular resistance genes present in Solanaceous species is represented by *Pto* which encodes a cytoplasmic serine/threonine protein kinase (Martin *et al.*, 1993; Riely *et al.*, 2001; Vleeshouwers *et al.*, 2001). Despite several attempts, we also could not assign Ecp2-recognition to these classes of *R* genes.

The search for putative *R* gene homologues by PCR-amplification or DNA-hybridisation is based on the presence of homologous DNA and consequently has its limitations as there is not always a direct link between protein structure and DNA sequence homology. For example, genes encoding proteins with a structure homologous to Cf-9 and Cf-2, like *Ve* (Kawchuk *et al.*, 2001) and *HcrVf* (Vinatzer *et al.*, 2001; Xu and Korban, 2002; Belfanti *et al*., 2004) are hardly homologous to the *Cf-9* or *Cf-2* genes. In the NBS-LRR class of genes DNA homology is generally low and some NBS-LRR genes present in the same species do not share any sequence homology, as is the case for *L. esculentum I2* and *Mi-1* (Ori *et al.*, 1997; Simons *et al.*, 1998; Rossi *et al.*, 1998; Vos *et al.*, 1998). Interestingly, the recognition of the *Pseudomonas syringae* effector protein AvrB is mediated by *RPM1* in *Arabidopsis thaliana* and by *Rpg1-b* in *Glycine max* (Ashfield *et al.*, 2004). Although *RPM1* and *Rpg1-b* both belong to the coiled-coil NBS-LRR class of *R* genes, they share only limited sequence similarity outside the conserved domains characteristic for this class. This lack of sequence homology may be the reason why we could not identify the *R*-gene that is involved in Ecp2 recognition in *Nicotiana* species while it still could be a distantly related *Hcr9*.

Ecp2-recognition is not crucial for the arrest of growth of *C. fulvum* in non-host plants

During growth of *C. fulvum* on tomato plants, *Ecp*2 expression is induced during intercellular growth (Wubben *et al.*, 1994). In the tested *Nicotiana* species, fungal growth was arrested as soon as the hyphae penetrated the stomata or at the stage the hyphae reached the substomatal cavity. No differences were observed between *N. paniculata* TW99, T101 (responding to Ecp2) and TW100 (not responding to Ecp2). This indicates

that *C. fulvum* lacks crucial pathogenicity factors enabling it to colonise the *Nicotiana* species studied. Alternatively, the *Nicotiana* species may produce antifungal compounds toxic to *C. fulvum*. Production of these compounds should be induced during fungal infection since *in vitro* growth of *C. fulvum* in apoplastic fluids isolated from different *Nicotiana* species is similar to *in vitro* growth in apoplastic fluids isolated from tomato leaves (results not shown).

We therefore conclude that Ecp2 production by *C. fulvum* is not crucial for the arrest of growth on Ecp2-responding *N. paniculata* and *N. tabacum*. It is unlikely that other proteins derived from *C. fulvum* can elicit HR responses in the studied *Nicotiana* species. Ecp2 was found to be the only elicitor protein that induced an HR in tobacco in a screen for HR-inducing cDNAs from *C. fulvum* grown in nitrogen-starved conditions (Takken *et al.*, 2000). We cannot exclude that additional proteins or non-proteinous compounds of *C. fulvum*, like cell wall components can induce non-HR based defence responses in *Nicotiana* species. However, no HR was induced upon infiltration of *Nicotiana* leaves with apoplastic fluid derived from a compatible interaction of a *C. fulvum* transformant that does not produce Ecp2 (results not shown).

Non-host resistance to *P. infestans* in parsley, tobacco and other species of the genus *Nicotiana* is diverse and exhibits various types of defence responses that culminate into an HR that can differ in intensity depending on the plant species tested (Hahlbrock *et al.*, 1995; Naton *et al.*, 1996; Kamoun *et al.*, 1998; Vleeshouwers *et al.*, 2000). However, no clear defence responses were observed in non-host resistance to *C. fulvum*. We cannot exclude the possibility that alternative responses are present. For example, the phytopathogenic fungus *Alternaria alternata* f.sp. *lycopersici* produces AAL toxins as the major virulence factor to colonise susceptible tomato plants. Resistance of tomato to AAL toxins is mediated by the *Asc-1* gene (Brandwagt *et al.*, 2000) which occurs at a low frequency (five out of the 68 tested *Nicotiana* species) in the *Nicotiana* genus (Brandwagt *et al.*, 2001a). In these species, however, AAL-toxin sensitivity is not absolutely correlated with susceptibility to *A. alternata* f.sp. *lycopersici*. Similarly, Ecp2-recognition in *Nicotiana* species occurs at a comparable frequency (four out of the 38 tested *Nicotiana* species) and lack of Ecp2-recognition in *Nicotiana* species does not lead to susceptibility to *C. fulvum*.

In conclusion, we have shown that HR-associated recognition of Ecp2 is mediated by a single dominant gene in *N. paniculata* and that absence of Ecp2-recognition did not lead to growth of *C. fulvum* on *Nicotiana* plants. However, based on PCR- and hybridisation analysis this gene is not homologous to known *Cf*-genes. Expression profiling of *N. paniculata* accessions differentiating for Ecp2-recognition by cDNA-AFLP may finally display the candidate gene that mediates the specific recognition.

EXPERIMENTAL PROCEDURES

Plant material

Seeds of the various *Nicotiana* species were obtained from the US National Plant Germplasm System (NPGS) at the National Seed Storage Laboratory (NSSL; at Fort Collins, CO, USA) from the Tobacco Collection (TOB) at the Crop Science Department, Oxford Tobacco Research Station, North Carolina State University, Oxford, NC, USA and the Botanical Garden of the University of Nijmegen, The Netherlands. As a host-plant for *C. fulvum* we used the near-isogenic line of tomato cultivar Moneymaker carrying no known functional *Cf* resistance genes (referred to as MM-Cf0) and the breeding line Ontario-7518 carrying the *C. fulvum* resistance gene *Cf-Ecp2* (referred to as tomato Cf-Ecp2; Kanwar *et al.*, 1980b; Haanstra *et al.*, 1999).

N. paniculata accessions were selfed (S1). $TW99_{S1}$ and $TW101_{S1}$ were reciprocally crossed to $TW100_{S1}$. The $TW99_{F1}$ and $TW101_{F1}$ progenies obtained were subsequently selfed, resulting in a F_2 progeny. $TW99_{F1}$ and $TW101_{F1}$ plants were also back-crossed to $TW100_{S1}$, resulting in a BC_1 population. To create a testcross population, the F_1 of the cross ($TW99_{S1}$ x $TW100_{S1}$) was crossed to $TW100_{S1}$.

PVX inoculation, agroinfiltration and Ecp2 protein infiltration

Plants were grown in the greenhouse in a daily regime of 16 hours of light at 21°C and eight hours of darkness at 19°C, at 60% relative humidity. Leaves of four- to six-week-old plants were inoculated as described by Hammond-Kosack *et al.* (1995) and Laugé *et al.* (1998a) with wild-type PVX, PVX::PR1a*Ecp*2 (*Ecp2* gene with PR1a signal sequence enabling secretion of Ecp2) or PVX::*Ecp2* (*Ecp*2 gene without signal sequence preventing secretion of Ecp2). Systemic or local HR was scored four days to two weeks after inoculation, depending on the appearance of symptoms on the different plants species. When the PVX-inoculated plants did not show any mosaic symptoms or HR, the tolerance or these plants for PVX was indirectly tested by inoculation of leaf sap of these plants containing PVX onto *Nicotiana* or tomato plants susceptible to PVX.

Agroinfiltration of *Nicotiana* species was essentially performed as described by Van der Hoorn *et al.* (2000). The binary expression constructs p*Cf-4*, p*Avr*4, (Van der Hoorn *et al.*, 2000) were used as control gene-pairs during agroinfiltration studies. *Agrobacterium tumefaciens* GV3101 cultures expressing the gene of interest were infiltrated in fully expanded *Nicotiana* leaves. HR was scored five days after infiltration.

A (N-terminally tagged) Ecp2 protein was produced in the *Pichia pastoris* expression system (Invitrogen) and affinity purified (Brandwagt, *in preparation*). Protein-dilution series were infiltrated in fully expanded *Nicotiana* leaves with or without 0.2% (v/v) Tween-80. Responses induced by Ecp2-recognition were scored seven days after infiltration.

Fungal inoculations and β-glucuronidase assay

An Ecp2-producing transgenic strain of *C. fulvum* producing β-glucuronidase (GUS) (Van den Ackerveken *et al.*, 1994), was subcultured on potato-dextrose agar at 22°C. Conidia from 10-day-old cultures were used to inoculate tomato MM-Cf0, tomato Cf-Ecp2, *N. tabacum* SR1 and *N. paniculata* accessions TW99, TW100 and TW101, as described by De Wit (1977). Samples were taken at 1, 4, 7, 12 and 18 days post inoculation and histochemical GUS assays were performed to visualise fungal growth. GUS activity assays were essentially performed as described by Jefferson *et al.* (1987) and Van den Ackerveken *et al.* (1994). HR symptoms were also visually scored.

Molecular genetic analyses

Genomic DNA was isolated from *Lycopersicon esculentum* species, *Solanum tuberosum* (potato), *Nicotiana sylvestris* TW136 and TW137, *N. tabacum* SR1 and Samsun NN , *N. paniculata* TW99, TW100 and TW101 and from 24 individuals of the F_2 population (TW101 x TW100), segregating for Ecp2-recognition (Haanstra *et al.*, 1999) as described by Van der Beek *et al.* (1992). *N. undulata* was only recently found to be responsive to Ecp2, this species was not included in these experiments. PCR-amplification was performed on 100ng of genomic DNA or 20 ng of plasmid DNA in 25µL or 50µL reaction volumes using *Supertaq* (HT Biotechnology) or *Pfu-Turbo* (Stratagene) DNA polymerase using annealing temperatures of 50°C to 55°C and extension times of one to three minutes, depending on the primer/template combination. The sequences of the oligonucleotides used in this study are listed in 6.

For gel blot analysis, five µg of genomic DNA was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Xba*I, or *Xho*I and fractionated on a 0.75% agarose gel and blotted onto a Hybond-N+ membrane (Amersham). The probes that were used for hybridisation (see Table 5) included the 3' region of the *Cf-9* gene [LRR18-27 and domains D, E, F and G, amplified from the cloned cDNA; domains are described in Jones *et al.* (1994)]; the 3' region of the *Cf-2* gene [LRR28 until domain G, amplified from the cDNA of *Cf-2.1*; domains are described in Dixon *et al.* (1996)]. In addition, the NBS-region of the *N* gene (Whitham *et al.*, 1994) was amplified from a plasmid containing the cloned *N*-gene using primer set NCF/NCR. The *Pto*-derived probe was PCR amplified from a plasmid containing *Pto* using primer set F1/R1 (Vleeshouwers *et al.*, 2001). Probes were labelled with ^{32}P and hybridisation of the Southern blots was performed overnight at 65°C. Membranes were washed at medium stringency at 65°C with 1x SSC (0.3 M NaCl, 0.03M NaAc), 0.1% SDS.

Table 6. Oligonucleotide sequences used for genomic DNA- and probe-amplification.

Oligonucleotide	Sequence (5' - 3') [a]	location
Hcr9START	ATGGRTTGTKTARAACTTRT	start codon of *Hcr9*
Hcr9STOP	CTAATATCTTTTCTTGTGCTTTTTCAT	stop codon of *Hcr9*
Hcr9C1F	CATGGGATGGMRTTSATTGTGAC	end of B domain of *Hcr9* [b]
Hcr9C1R	CATWGTGGGATTGTYCCCTCC	in LRR17 of *Hcr9* [b]
HEseq4f	GAACAATCCCACTATGTTTGGG	in LRR18 of *Hcr9* [b]
Hcr2START	ATGATGATGGTTTCTAGAAAAG	start codon of *Hcr2*
Hcr2STOP	CTAGAAGTGATTATTTCTTCTTC	stop codon of *Hcr2*
Hcr2LRR28F	GGCAGAAACAATCTGGAGG	in LRR28 of *Hcr2* [c]
Hcr2LRR28R	CCTCCAGATTGTTTCTGCC	in LRR28 of *Hcr2* [c]
NCF	GGAATGGGGGGAGTCGG	upstream NBS-domain of *N* (*N. glutinosa)*
NCR	AACATCTCTTGTTGTTTGGGC	downstream NBS-domain of *N*
PtoF1 [d]	CAAATTCGATAAATGATGC	amino acid 9-15 in Pto (*L. esculentum*)
PtoR1 [d]	CCGAAAGAATAAACATCAG	amino acid 222-228 in Pto, upstream of activation domain

(a) R = A or G; K = G or T; M = A or C; S = C or G; W = A or T

(b) domains are described in Jones *et al.* (1994)

(c) domains are described in Jones *et al.* (1996)

(d) primers adapted from Vleeshouwers *et al.* (2001)

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Chapter 6

Ecotilling of *Cladosporium fulvum* shows a very high mutation rate in Avr and Ecp elicitor proteins

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ABSTRACT

Resistance against the fungal pathogen *Cladosporium fulvum* is initiated by recognition of avirulence (Avr) proteins by its host plant tomato. DNA sequence analysis of the Avr proteins (so-called race-specific elicitors) Avr2, Avr4, Avr4E and Avr9 have revealed that the change from avirulence to virulence is associated with DNA mutations in the Avr coding regions. The high frequency of these mutations are most likely the result of high selection pressure caused by the frequent use of matching *Cf* resistance genes in commercial tomato lines. However, the *Cf-Ecp* resistance genes have rarely been employed and previous research showed that no variation was found the Ecp elicitors. In the present study, the Ecotilling method was performed in strains of *C. fulvum* that have been collected world-wide to compare the sequence variation in Avr and Ecp elicitor encoding genes while the variation in ribosomal internal transcribed spacers (ITS) was used as evolutionary clock. No polymorphisms in ITS sequences were observed. Silent mutations in *Avr*s occurred more frequently. However, the very fast majority of the mutations in Avr proteins were associated with virulence and indicate a high selection pressure in *C. fulvum Avr* elicitor genes. In *Ecp* elicitor genes, however, mutations occurred rarely and were not associated with virulence. These results show a very high mutation rate in elicitor proteins and confirm the lack of selection pressure on the *Ecp* genes by *Cf-Ecp* resistance genes.

INTRODUCTION

There is a long history in breeding for resistance against the fungal pathogen *Cladosporium fulvum* in tomato (*Lycopersicon esculentum*). Soon after the introduction of a *Cf* resistance gene in tomato cultivars, the virulence pattern of the fungus changed and, subsequently, the resistance often became ineffective. Intensive studies have been performed to find novel *Cf* genes in wild *Lycopersicon* species that were followed by the introduction of new *Cf* genes into tomato cultivars (Kerr *et al*., 1971; Kanwar *et al*., 1980a,b). Since also many different races of *C. fulvum* have been characterised (Lindhout *et al*., 1989), the interaction between tomato and *C. fulvum* has become one of the best-studied plant-pathogen interactions that fits the gene-for-gene relationship (Joosten and De Wit, 1999). This model postulates that for each gene determining resistance in the host, there is a corresponding gene conditioning avirulence of the pathogen (Flor, 1942).

The resistance genes *Cf-2*, *Cf-4*, *Hcr9-4E*, *Cf-5*, *Cf-9* and *9DC* have been isolated over the last ten years (Dixon, *et al*., 1996, 1998; Jones *et al*., 1994; Takken *et al*., 1998; Thomas, *et al*., 1997; 1998; Van der Hoorn *et al*., 2001a), whereas detailed

characterisation of *Cf-Ecp1*, *Cf-Ecp2*, *Cf-Ecp3*, *Cf-Ecp4* and *Cf-Ecp5* is still in progress (Haanstra *et al*., 1999, 2000, Yuan *et al*., 2002; De Kock and Brandwagt, unpublished data, CHAPTER 2, 3, and 4). The *Cf-2*, *Cf-4*, *Cf4E* and *Cf-9* resistance genes are widely used in European tomato breeding programs (P. De Wit, P. Lindhout, *pers. comm*.) However, the *Cf-Ecp* genes have rarely, or not been used in commercial tomato breeding programs (P. De Wit, P. Lindhout, *pers. comm*.). Only *Cf-Ecp2* was found in certain Canadian breeding lines (Kanwar *et al*., 1980b, Laugé *et al*., 1998a; Haanstra *et al*., 1999).

Avirulence (Avr) factors of *C. fulvum* elicit an hypersensitive response (HR) in tomato harbouring the corresponding *Cf* resistance gene. To date, several *Avr* genes from *C. fulvum* have been cloned that act as an elicitor of host defence on resistant plants and encode cysteine-rich proteins that are abundantly secreted by *C. fulvum* during colonisation of tomato leaves. These proteins do neither share sequence homology with each other, nor with any sequence present in public sequence databases. Four of these elicitors, Avr2, Avr4, Avr4E and Avr9, are race-specific and correspond to the four *Cf* genes widely applied in tomato breeding (Luderer *et al*., 2002a; Joosten *et al*., 1994; Westerink *et al*., 2003; Van Kan *et al*., 1991) Elicitor-recognition by the host plants leads to avirulence of strains of *C. fulvum* as the HR prevents further fungal growth and the plant becomes resistant. As a result of decades of selection pressure in the fungus, wild-type Avr factors are produced only by certain *C. fulvum* strains. Detailed analysis of naturally occurring strains of *C. fulvum* that are virulent on tomato genotypes containing a *Cf* resistance gene revealed that the fungus employs distinct mechanisms to avoid recognition by the host plant (summarised in Table 1). The change from avirulence to virulence is generally associated with DNA mutations in the Avr coding regions (Joosten *et al*., 1997; Luderer *et al*., 2002a; Talbot *et al*., 1991; Van Kan *et al*., 1991; Westerink *et al*., 2003). The absence of one or more wild-type Avr factors in a *C. fulvum* strain, with no visible loss of pathogenic fitness, suggests that these Avr factors are probably dispensable (Joosten and De Wit, 1999). However, for Avr4 the putative intrinsic function is preserved in some mutant alleles (Van den Burg *et al*., 2003). Wild-type Avr4 constains a functional chitin-binding domain that is thought to protect the fungus against tomato chitinases. While evasion of *Cf-4*-mediated resistance appears to result from instability and protease sensitivity of Avr4 mutant proteins, the unstable Avr4 mutants are still capable of binding to chitin (Van den Burg *et al*., 2003).

In contrast to the race-specific Avr factors, the other *C. fulvum* elicitors, extracellular proteins Ecp1, Ecp2, Ecp4, and Ecp5, as well as Ecp3, for which the encoding gene has not yet been identified, are secreted by all strains of *C. fulvum* that have been analysed up till now (Van den Ackerveken *et al*., 1993; Laugé *et al*., 1998a,b, 2000). Opposed to the Avr proteins, no DNA modifications have been found so far in the *Ecp* genes of naturally occurring strains of *C. fulvum* that affect *Cf-Ecp*-mediated resistance. This might be due to

lack of selection pressure on the pathogen to overcome *Cf-Ecp* mediated resistance, as the *Cf-Ecp*s have likely not yet been introduced yet in commercial tomato cultivars. On the other hand, as all strains of *C. fulvum* analysed so far secrete Ecp1 and Ecp2, disruption or modification of the encoding gene was thought to reduce the pathogenic fitness of the fungus. Indeed, based on a single gene-replacement transformants, *Ecp2* appeared required for colonisation and sporulation of *C. fulvum* on mature tomato plants (Laugé *et al*., 1997). A single *Ecp1*-deficient strain sporulated less abundantly than the wild-type strain on mature tomato (Laugé *et al*., 1997). Although based on a limited number of transformants, both Ecp1 and Ecp2 seemed to be required for pathogenicity of *C. fulvum* on tomato. However, gene-specific knock-down by RNA interference (RNAi) showed that none of the Ecps, nor AvrE factors individually contribute to pathogenic fitness as all RNAi strains appeared fully virulent (B. Brandwagt, unpublished data). However, both *Ecp1*- and *Ecp2*-deficient strains induce plant defence-associated responses more quickly and to higher levels than wild-type strains, suggesting that both Ecps are involved in suppression of host defence-associated responses during colonisation (Laugé *et al*., 1997).

Based on our current knowledge, there exist numerous mutations in the Avr proteins. In contrast, the Ecp proteins seem to be conserved. The question remains whether silent mutations occur in Avr and Ecp proteins in the same frequency as effective mutations are found in Avr protein. Additionally, not all *C. fulvum* strains have been tested genotypically and phenotypically for the sequence of the complete set of the known elicitor encoding genes that they carry. Therefore, we started the identification of polymorphisms in genes encoding elicitors of natural strains of *C. fulvum* of a world-wide collection. The aim was to get a complete picture of the sequence variation in the various *Avr*s and *Ecp*s among the *C. fulvum* population and compare the frequency of silent mutations and mutations affecting avirulence.

We exploited the mutation detection technology used in Targeting Induced Local Lesions in Genomes (TILLING). TILLING is a low-cost, high-throughput reverse genetics method that combines random chemical mutagenesis with PCR-based screening for DNA mutations in gene regions of interest (Colbert *et al.*, 2001; McCallum *et al.*, 2000a,b). The method was adapted by Comai *et al*. (2004) for detecting multiple types of natural polymorphisms in natural *Arabidopsis thaliana* accessions, a strategy that referred to as 'Ecotilling'. The genomic region of interest is PCR-amplified with fluorescent primers from a queried accession and mixed with the fluorescent PCR-amplified region of a reference wild-type accession. After heating and annealing, nucleotide changes are identified by enzymatic digestion of heteroduplexes with the mismatch cleavage endonuclease CEL I and subsequent detection of cleaved fluorescent products on denaturing polyacrylamide gels using LI-COR DNA analysers (Kulinski *et al*., 2000; Oleykowski *et al*., 1998).

Table 1. Overview of mutations present in coding regions of *Avr2*, *Avr4*, *Avr4E*, *Avr9*, *Ecp1*, *Ecp2*, *Ecp4* and *Ecp5* in different *C. fulvum* strains and the effect of the mutation on resistance in tomato mediated by the corresponding *Cf* gene.

Elicitor	# Strains analysed	Mutation in gene	Codon position in ORF	Mutation in protein	Loss of *Cf*-mediated resistance
Avr2 [a]	15	No mutation	-	No mutation	no
	4	C to T	67	$Gln^{67}Stop$	yes
	11	ΔA	23	Frame shift	yes
	1	ΔC	24	Frame shift	yes
	1	Δ 176 bp	58	Large deletion	yes
	2	ΔT	72	Frame shift	yes
	7	+A	23	Frame shift	yes
	1	+A	40	Frame shift	yes
	2	+Transposon	19	Insertion of 5 kb	yes
Avr4 [a]	28	No mutation	-	No mutation	no
	1	ΔC	42	Frame shift	yes
	2	G to T	64	$Cys^{64}Tyr$	yes
	1	C to T	66	$Thr^{66}Ile$	yes
	2	T to C	67	$Tyr^{67}His$	yes
	4	G to T	70	$Cys^{70}Tys$	yes
	6	G to T	109	$Cys^{109}Tyr$	yes
Avr4E [a]	9	No mutation	-	No mutation	no
	16	T to C; T to C	82 and 93	$Phe^{82}Leu$; $Met^{93}Thr$	yes
	30	Deletion of ORF		No protein	yes
Avr9 [a]	43	No mutation	-	No mutation	no
	12	Deletion of ORF		No protein	yes
Ecp1	37	No mutation	-	No mutation	no
Ecp2	36	No mutation	-	No mutation	no
	2	G to T	64	$Arg^{64}Ile$	no
Ecp4	29	No mutation	-	No mutation	no
	7	G to T	90	$Gln^{90}Val$	n.d.[b]
Ecp5	23	No mutation	-	No mutation	no

(a) Data of *Avr2*, *Avr4*, *Avr4E*, and *Avr9* partially or completely adapted from Joosten *et al.*, 1997; Luderer *et al.*, 2002a; Talbot *et al.*, 1991; Van Kan *et al.*, 1991; Westerink *et al.*, 2003; see text for details.

(b) Not determined yet.

In contrast to the absolute conservation of the internal transcribed spacers of the rDNA genes, silent mutations in *Avr*s occurred more frequently. However, the very fast majority of the mutations in Avr proteins were associated with virulence and indicate a high selection pressure in *C. fulvum Avr* elicitor genes. In *Ecp* elicitor genes, however, mutations were observed rarely and were not associated with virulence. These results show a very high mutation rate in elicitor proteins and confirm the lack of selection pressure on the *Ecp* genes by *Cf-Ecp* resistance genes.

RESULTS AND CONCLUSIONS

We adapted the Ecotilling method described by Comai *et al*. (2004) for detecting multiple types of natural polymorphisms in strains of *C. fulvum* that previously have been collected world-wide, including Europe, North America, South America, Japan, Australia and New Zealand. An overview of the approach is depicted in Figure 1. A *C. fulvum* strain with the race 0 phenotype was used as wild-type reference strain to which the other *C. fulvum* strains were compared. First, the reliability of the mutation-detection method was validated by screening for mutations that have already been described in *Avr2* and *Avr4* (Luderer *et al*., 2002a; Joosten *et al*., 1994 and 1997). Subsequently, *Ecp1*, *Ecp2*, *Ecp4* and *Ecp5* were screened for mutations and finally, the sequence variation in the internal transcribed spacer (ITS) 1 and 2 of the ribosomal RNAs was studied. These ITS sequences evolve relatively fast and are frequently used for comparing species and closely related genera (Soltis and Soltis, 1999).

[Avr2] All single nucleotide polymorphisms (SNPs) reported for *Avr2* were confirmed in the tested reference strains. In a yet uninvestigated strain (#62) virulent on *Cf-2* plants the *Avr2* ORF was found to contain a 176 bp deletion that results in a truncated protein (Figure 2 and Table 1). In total, eight mutations of different nature, all resulting in the production of mutant Avr2 isoforms, were found. Additionally, seven *C. fulvum* strains were found that contain a non-effective mutation (ΔTGA) in the intron of *Avr2*.

[Avr4] All known *Avr4* mutations could be reconfirmed by the Ecotilling mutation-detection method (Table 1), except for mutations present in two strains. In these strains, the expected mutations could yet be confirmed by sequence analysis. In four strains avirulent on *Cf-4* plants and producing a functional Avr4 elicitor, a silent mutation (G to A) was found in the promoter region of the *Avr4* gene (Figure 2). In total, six different mutations, mainly nucleotide substitutions that abolished wild-type Avr4 production and recognition in *Cf-4* tomato plants, were reconfirmed in the *Avr4* ORF. Based on the results obtained for *Avr2* and *Avr4*, the Ecotilling method proved to be a reliable method for the screening of known and unknown mutations in genomic regions of *C. fulvum*.

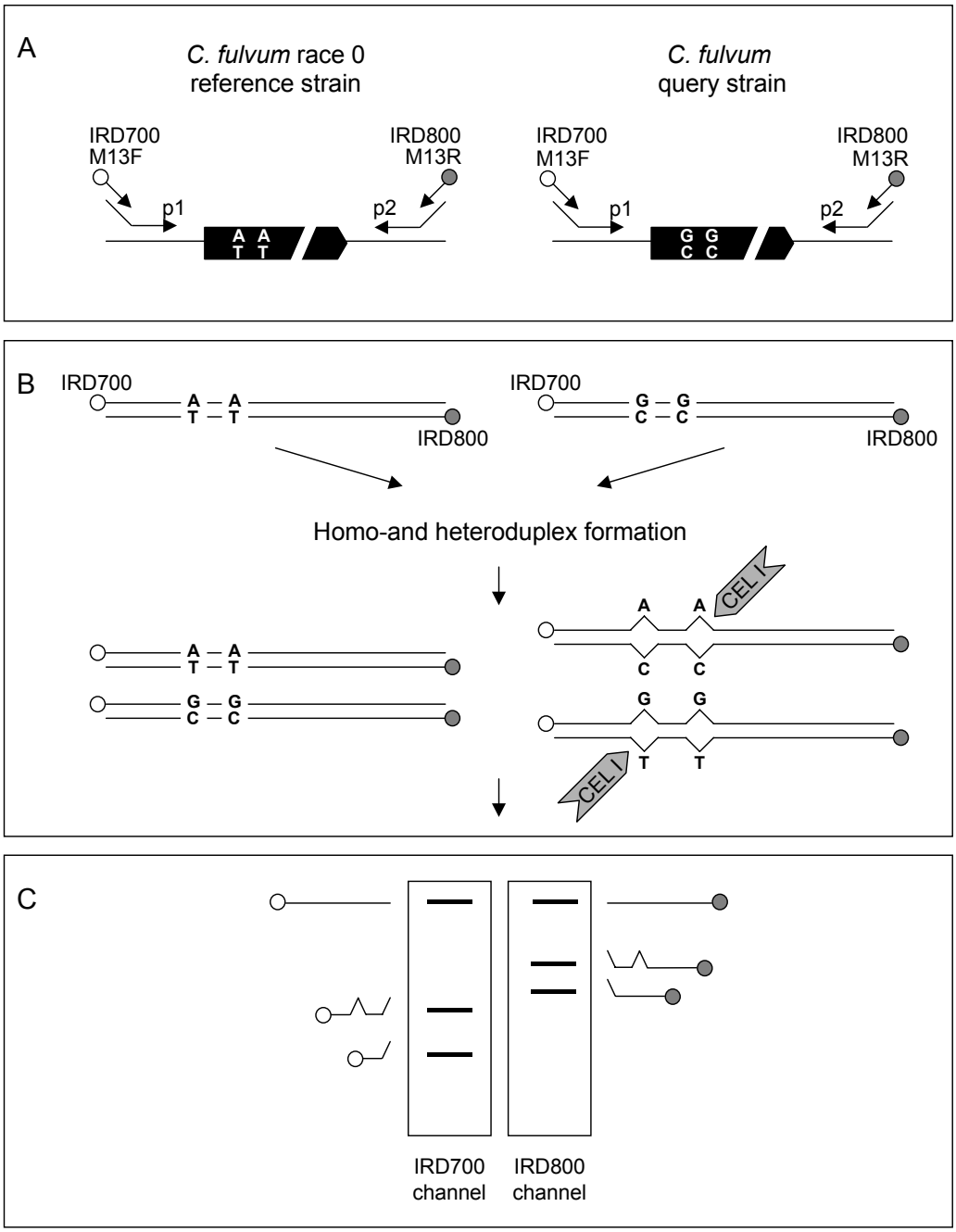

Figure 1. Schematic representation of the Ecotilling method for the detection of multiple types of polymorphisms in *C. fulvum*. (**A**) The genomic region of interest from a reference wild-type strain and a query strain is PCR-amplified using primers p1 and p2 and subsequently labelled by a nested PCR using an IRD-fluorescent labelled M13 primer set (M13F and M13R). The ends of the amplified DNA are labelled differentially with either the IRD700 dye (on the 5' end) or IRD800 (on the 3' end). The black arrowed box indicates the gene of interest with two polymorphic sites. (**B**) After mixing both labelled PCR products, heating and annealing, nucleotide changes are identified by enzymatic digestion of heteroduplexes with the CEL I mismatch cleavage endonuclease. (**C**) The cleaved fluorescent products are subsequently detected on an electrophoretic LI-COR gel analyser. The two fluorescent dyes are detected in different channels, and two images are generated for each electrophoretic run. Candidate polymorphic sites identified in the IRD700 channel (showing the 5'-labelled cleavage products) can be confirmed in the IRD800 channel, which shows the complementary cleavage products that are labelled on the 3'-end.

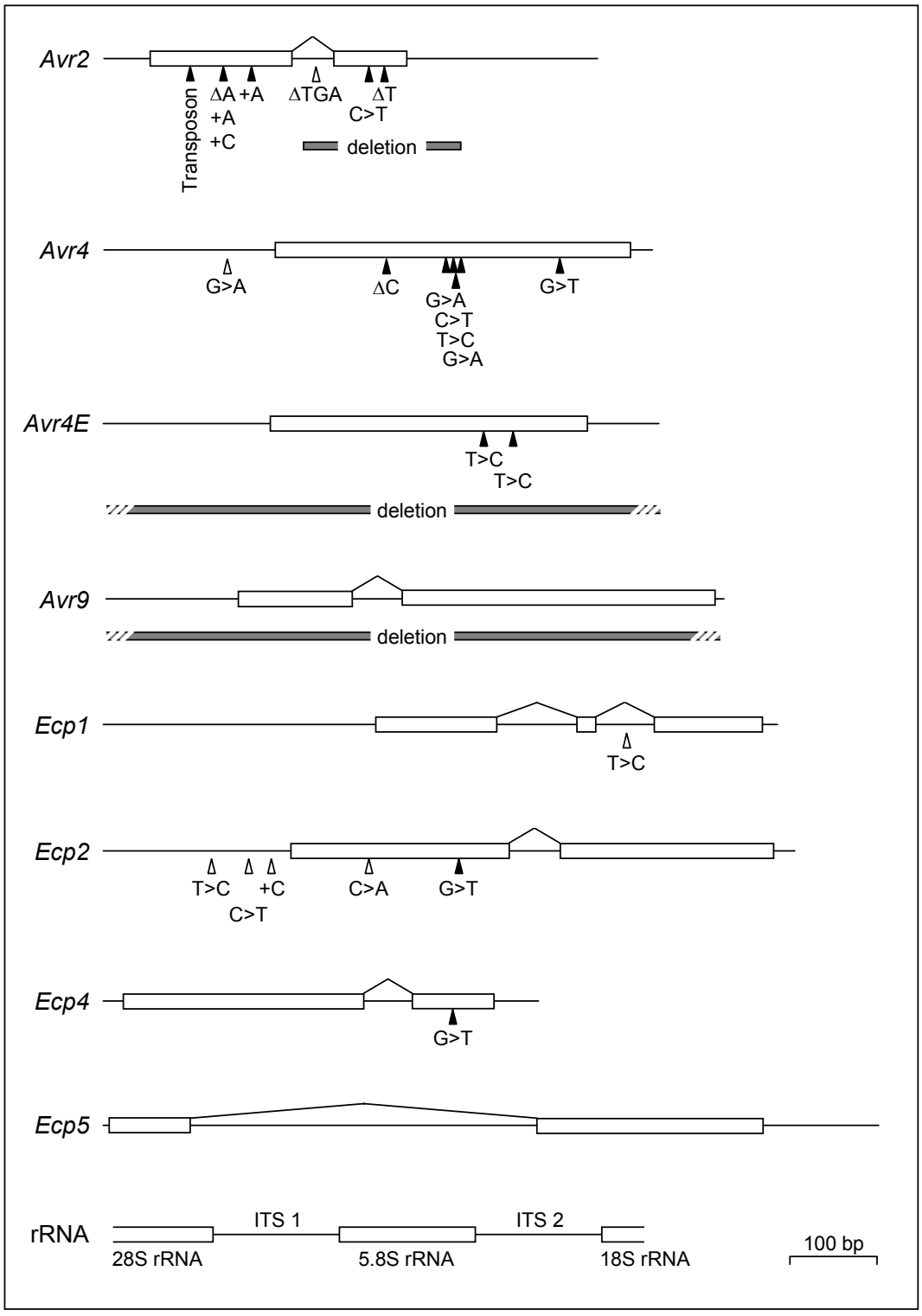

Figure 2. Overview of genomic regions, including exons and introns, studied for sequence polymorphisms in *C. fulvum* and the discovered mutations. Positions and details of mutations in the elicitors *Avr2*, *Avr4*, *Avr4E*, *Avr9*, *Ecp1*, *Ecp2*, *Ecp4* and *Ecp5* and internal transcribed spacers (ITS) of the region encoding the ribosomal RNAs are indicated with triangles (open triangle: silent- or non effective mutation; black triangle: mutation resulting in a frame shift or amino-acid change). Large deletions are indicated by a grey bar. Data of *Avr2*, *Avr4*, *Avr4E*, and *Avr9* partially or completely adapted from Joosten *et al*., 1997; Luderer *et al*., 2002a; Talbot *et al*., 1991; Van Kan *et al*., 1991; Westerink *et al*., 2003; see text for details.

The Ecotilling method was subsequently used to investigate the sequence variation in *Ecp*s. For various *Ecp*s, particular strains did not yield a PCR product. It is unknown whether this is the result of a less optimal PCR, or the absence of the gene of interest as is the case for *Avr4E* and *Avr9* Table 1 and Figure 2). Additional experiments, including DNA gel blot analysis have to prove whether a wild-type or mutant *Ecp* allele is present, or whether the gene is completely absent.

[Ecp1] The *Ecp1* coding region is conserved in 37 tested strains (Table 1 and Figure 2). However, four strains (strain #2, race 2; and strains #19, #20, and #21, all race 5) contained a T to C substitution in the second intron (Figure 2). The *Ecp1* gene was cloned from *C. fulvum* race 5 (Van den Ackerveken *et al*., 1992) and differs from the wild-type sequence of race 0. A sexual cycle has never been observed for *C. fulvum,* suggesting that the *C. fulvum* population consist of single clonal lineages (Joosten and De Wit, 1999). Therefore, it can be concluded that in these four strains the nucleotide substitution in the *Ecp1* coding region predated the mutations in the *Avr2* and *Avr5* alleles, respectively.

[Ecp2] Thirty-six of the 38 tested *C. fulvum* strains contained a conserved *Ecp2* genomic region. However, in two strains (#42 and #62) identical multiple SNPs were found (Table 1 and Figure 2). Three mutations were in the promoter region and one mutation (C to A) was a silent mutation in the *Ecp2* ORF. The fifth mutation (G to T) resulted in the substitution of an arginine residue at amino acid position 64 by an isoleucine residue. Ecp2 was immunodetected in apoplastic fluid from susceptible tomato inoculated with these *C. fulvum* strains (Laugé *et al*., 1998a). These apoplastic fluids produced an HR in *Cf-Ecp2* tomato plants. Moreover, strains #42 and #62 showed a wild-type virulence on susceptible tomato. It can therefore be concluded that theses mutations do not harm, neither benefit *C. fulvum*. Strain #42 is a race 3 originating from Canada, whereas strain #62 is a race 2.6.7.9 which was collected in New Zealand. This indicates that the mutations found in the *Ecp2* genomic region predate the mutations resulting in the race-specificity of these two strains. In contrast, together with the results described for *Ecp1*, these results might be explained by the presence of gene exchange between *C. fulvum* strains. However, a sexual cycle has never been observed for *C. fulvum*.

[Ecp4] In seven of the 36 studied *C. fulvum* strains, as a result of a G to T mutation, the glutamine residue at amino acid position 90 in Ecp4 was changed to a valine residue (Table 1 and Figure 2). It is presently unknown whether this mutation affects the HR-inducing activity of Ecp4 on *Cf-Ecp4* tomato plants. Four of the seven strains were included in the research described by Laugé *et al*. (1998a). As these strains showed a wild-type virulence on susceptible tomato, the $Gln^{90}Val$ mutation is not thought to be essential for the virulence of *C. fulvum.*

[Ecp5] Twenty-three strains could be tested for mutations in the *Ecp5* coding region. In the genomic sequence of *Ecp5* a large intron (397 bp) was identified, but all strains contain a

wild-type ORF, indicating that *Ecp5* is very conserved (Table 1 and Figure 2). Analysis of the remaining twenty-one strains was not possible as there was no PCR product produced.

[ITSs]The genes encoding the ribosomal RNAs are highly conserved, whereas the ITS regions are much more rapidly evolving (Soltis and Soltis, 1999). Therefore, it was not expected to identify any mutations in the 5.8S coding region and fragments of the 18S and 28S rDNA sequences but mutations in the ITSs would be expected. However, in all tested strains the studied region was fully conserved (Figure 2) and confirms the results of Curtis *et al*. (1994).

Sequence variation in *Avr4E* and *Avr9* was not investigated in the present study. For comparison of the mutations in *Avr4E* and *Avr9* we used the data previously described (Westerink *et al*., 2003; Talbot *et al*., 1991; Van Kan *et al*., 1991). It would be interesting to apply the Ecotilling method also on these two elicitor genes of *C. fulvum*. It can not be excluded that additional (silent) mutations will be found.

It has to be noted that all tested *C. fulvum* strains have been collected from tomato fields and greenhouses and that natural *C. fulvum* strains originating from South-America, the site of origin of *Lycopersicon* species, are unfortunately not available yet. The sequence variation present in the *Avr*s, *Ecp*2 and *Ecp*4 is in contrast to the full conservation of the ITS sequences. Dynamics in *Avr* alleles therefore support the selection pressure imposed on *C. fulvum* as a result of the wide deployment of *Cf-4* and *Cf-9* resistance genes over the *Lycopersicon* genus (M. Kruijt, *pers. comm.*) and the frequent deployment of *Cf-2 Cf-4*, *Hcr9-4E* and *Cf-9* in tomato breeding. However, it is conspicuous that the fast majority of the mutations that are found in the *Avr*s affect *Cf*-mediated resistance. On the other hand, the lack of comparable numbers of silent mutations in *Avr*s is a very interesting finding. Mutations that result in Ecp isoforms are very rare and mutations affecting Ecp elicitor characteristics appear to be absent. These results differ strongly from the results obtained for the Avr proteins. Additionally, these results indicate that *Cf-Ecp*-mediated resistance is not broken yet. However, the *Cf-Ecp* genes have rarely, or not been used in breeding programs yet and the frequency of *Cf-Ecp* genes occurring in individual *Lycopersicon* accessions is presently unknown. As a result, assuming a selective advantage for *Ecp* isoforms without elicitor activity is currently speculative.

Dynamics of the *Ecp* genes are difficult to predict after introduction of *Cf-Ecp* genes in breeding programs and the selection against *Cf-Ecp*-mediated resistance is initiated. However, since the Ecps were found to be as redundant for virulence as the Avrs (B. Brandwagt, unpublished data), the Ecps are likely to accumulate mutations avoiding recognition by corresponding *Cf-Ecp* genes as well.

Table 2. Oligonucleotide sequences used for genomic DNA PCR-amplification.

Gene	forward primer p1 (5' - 3')[a]	reverse primer p2 (5' - 3')[b]
Avr2	[M13F]CATCAGCATATCCTCTTCCATCC	[M13R]CAGTACGTTCAAAAGCAGATAAGG
Avr4	[M13F]GTACACGAGCCACAATAAG	[M13R]CACTGCGTTATCCCCTTTCT
Ecp1	[M13F]ACCAGGATAAGTTCGCAGTC	[M13R]GCCTCCGCGATGCTTCCT
Ecp2	[M13F]GTCCAAGGATCGTGTCTCAAG	[M13R]TTCTAGCAAACCCGTCTGA
Ecp4	[M13F]AACCTAACATCACAATCTTCAA	[M13R]TTGCTCAAGCCCAGACAG
Ecp5	[M13F]TAGCTGATATTATGAACACTTT	[M13R]CGCGTCGCCTGATAGAT
rRNA	[M13F]GCTTAAGTTCAGCGGGTATCC	[M13R]CGGCAACGACCACCCAGG

(a) M13F target sequence (TCCCAGTCACGACGTTG) is preceding the 5' end of the forward primer
(b) M13R target sequence (GGATAACAATTTCACACAGG) preceding the 5' end of the reverse primer

EXPERIMENTAL PROCEDURES

Forty-four *C. fulvum* strains have previously been isolated from commercially cultivated tomato in different parts of Europe, Canada, USA, South America, Australia, New Zealand and Japan and stored at the Laboratory of Phytopathology, Wageningen University, The Netherlands. *C. fulvum* strains were cultured on potato-dextrose agar at 22°C. Conidia from 10-day-old cultures were cultured for 10 days in liquid B5 medium and genomic DNA of *C. fulvum* was isolated according to the procedure described by Van Kan *et al*. (1991). Following DNA isolation, genomic regions to be analysed were PCR-amplified and fluorescently labelled in a two-step nested-PCR approach (Figure 1). In the first PCR step genomic regions encoding the elicitors *Avr2* (AJ421628), *Avr4* (Y08356), *Ecp1* (Z14023), *Ecp2* (Z14024), *Ecp4* (AJ271890), and *Ecp5* (AJ271891) were PCR-amplified using forward and reverse primers p1 and p2 (Table 2). Primers p1 and p2 have a M13F or M13R universal primer extension which enables fluorescent labelling in a second nested PCR (Figure 1). Additionally, the genomic region encoding the partial 28S ribosomal RNA gene, internal transcribed spacer (ITS) 1, 5.8S ribosomal RNA gene and ITS-2 and the partial 18S ribosomal RNA gene (AF393701) was studied. The organisation of genomic regions, including the positions of exons and introns is indicated in Figure 2. PCR-amplification was performed in 25 cycles on 50ng of genomic DNA in a 15µL reaction volume using *Supertaq* (HT Biotechnology) DNA polymerase using annealing temperatures of 47°C to 55°C (depending on the primer combination) and an extension at 72°C for one minute. Amplification products were separated on a 1% agarose gel to analyse size and quantity. Ten ng of PCR product from the *C. fulvum* reference strain (race 0) and 10 ng of PCR product from the *C. fulvum* queried strain were subsequently mixed and used as a template for the nested PCR using IRD700-labelled M13F (5'-tttcccagtcacgacgttg-3') and IRD800 labelled M13R (5'-ggataacaatttcacacagg-3') primers (in 25µL reaction volume, annealing temperature 50°C, 35 cycles) Figure 1). Heteroduplex formation was performed by heating the nested PCR products for 10 minutes at 95°C and linearly cooling to 4°C in 10 minutes. Amplification products and heteroduplexes were separated on a 1% agarose gel to analyse quality and quantity.

Digestion of the heteroduplexes at mismatch sites was performed with the CEL-1-like endonuclease Surveyor Nuclease according to suppliers protocol (Transgenomic, Omaha, USA). The cleaved fragments were size- separated and detected on a LI-COR 4200 automated DNA sequencer, essentially following the

method published by Myburg *et al* (2001). For the reference strain #1 (race 0), all amplified genomic regions were completely sequenced and the obtained sequence was used as reference sequence. The detected polymorphisms were further confirmed by DNA sequencing (BaseClear, Leiden, The Netherlands).

Chapter 7

General discussion

Extracellular membrane-bound leucine-rich repeat resistance proteins in plants

Co-autheurs: Marco Kruijt, and Pierre J.G.M. de Wit

INTRODUCTION

Plants are attacked by many pathogenic organisms including viruses, bacteria, fungi, and nematodes. Plants resist 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).

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 have an intercellular localisation (Martin *et al.*, 2003). Class 4 mainly comprises the tomato Cf proteins, which have an extracellular LRR domain, a single transmembrane (TM) domain, and a small cytoplasmic tail (reviewed by Joosten and De Wit, 1999). This class of proteins is referred to as receptor-like proteins (RLPs). Finally, class 5 consists of the Xa21 protein from rice and the Arabidopsis FLS2 protein, which, in addition to an extracellular LRR domain and a TM domain, have a cytoplasmic serine/threonine kinase domain (Gomez-Gomez and Boller, 2000; Song et al., 1995). This class of proteins is referred to as receptor-like kinases (RLKs). A few R proteins do not fall into any off 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).

In this review we focus on proteins from class 4, with emphasis on the well-studied *Cf* proteins from tomato that mediate resistance against the biotrophic fungus *Cladosporium fulvum* (Joosten and De Wit, 1999). Cf proteins from tomato have long been

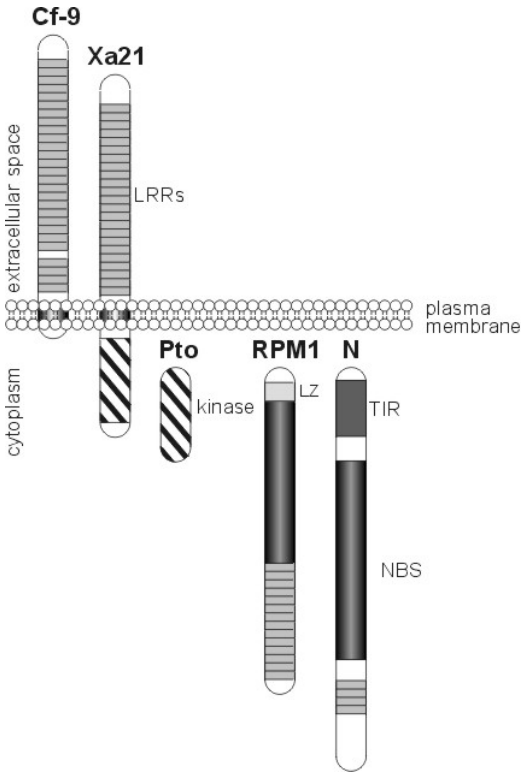

Figure 1. Schematic representation of the five major structural classes of plant R proteins. One R protein of each class is represented. Cf-9, *Cladosporium fulvum* resistance protein *from Lycopersicon pimpinellifolium* (currant tomato); Xa21, *Xanthomonas oryzae* pv. *oryzae* resistance protein from *Oryza sativa* (rice) ; Pto, *Pseudomonas syringae* pv. *tomato* resistance protein from *L. pimpinellifolium*; RPM1, resistance proteins against *P. syringae* pv. *maculicola* from *Arabidopsis thaliana*; N, resistance protein against Tobacco Mosaic Virus from *Nicotiana tabacum* (tobacco) (Grant *et al*., 1995; Jones *et al*., 1994; Martin *et al*., 1993; Song *et al*., 1995; 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.

the only known RLP resistance proteins in plants. However, recent studies have identified Cf-like resistance proteins 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 RLPs mediate disease resistance, and the role of RLPs in other biological processes.

Characteristics of RLPs 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 RLPs involvend in resistance. 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 in the extracellular space. De Wit and Spikman (1982) showed that specific elicitor proteins are present in the apoplastic fluids of infected plants. From these apoplastic fluids several elicitor proteins have been isolated from which the corresponding genes have been cloned

and characterised (reviewed by Joosten and De Wit, 1999; Luderer *et al.,* 2002a; Westerink *et al.,* 2003). 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. Many *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*., 2002a; 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 of *C. fulvum* elicitors comprises the Extracellular proteins (Ecps), which are secreted by all known *C. fulvum* strains during infection, and race-specificity has not yet been observed (reviewed by Joosten and De Wit, 1999, Laugé *et al*., 2000, De Kock *et al*., CHAPTER 6). Matching *Cf* genes, designated *Cf-Ecp* genes*,* have been identified in wild tomato species (Laugé *et al*., 1998a, 2000). Ecp1 and Ecp2 may play an important role in pathogenicity and the suppression of host defence responses (Laugé *et al*., 1997). Therefore, it was suggested that the corresponding *Cf-Ecp* genes are more durable (Laugé *et al*., 1998a). Sequence variation within the *Ecp* genes is largely absent (De Kock *et al*., CHAPTER 6). This reflects the fact that the *Cf-Ecp* genes have not been deployed in large-scale breeding programs, and hence, there is no selection pressure for virulence. Dynamics of the *Ecp* genes are difficult to predict as soon as *Cf-Ecp* genes are widely used in breeding programs and selection against *Cf-Ecp*-mediated resistance is initiated. Since the Ecps were tested to be as redundant for wild-type aggressiveness as the Avrs (B. Brandwagt, unpublished data), the Ecps will likely accumulate mutations rendering the corresponding *Cf-Ecp* genes.

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*., 1998b; Parniske *et al.*, 1997; Takken *et al.,* 1998; Thomas *et al.,* 1997; Van der Hoorn *et al.,* 2001a). 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; Jones *et al*., 1993; Haanstra *et al*., 1999, 2000a; Kruijt *et al*., unpublished data; Parniske *et al.*, 1997; Takken *et al*., 1998; Yuan *et al*., 2002). 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 from tomato thus far comprises two near-identical functional *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 in the susceptible tomato cultivar MoneyMaker Cf0 carries two *Hcr2*s with no known function in *C. fulvum* resistance (Dixon *et al*., 1998). The *Cf-2* cluster, which originates from *L. pimpinellifolium*, comprises three *Hcr2*s, including the two functional *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*., 2002a). The two *Cf-2* genes are likely the result of a recent duplication event. The *Cf-5* cluster from *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 from *Hcr2-5C* only by a single amino acid flanking these two LRRs. *Hcr2-5D* is expressed, but despite its high homology with *Cf-5*, does not confer resistance to *C. fulvum* races 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 must have occurred before speciation (Dixon *et al.*, 1998).

All *Hcr9*s are likely derived from a single *Hcr9* progenitor gene, as became evident from 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 with unknown 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

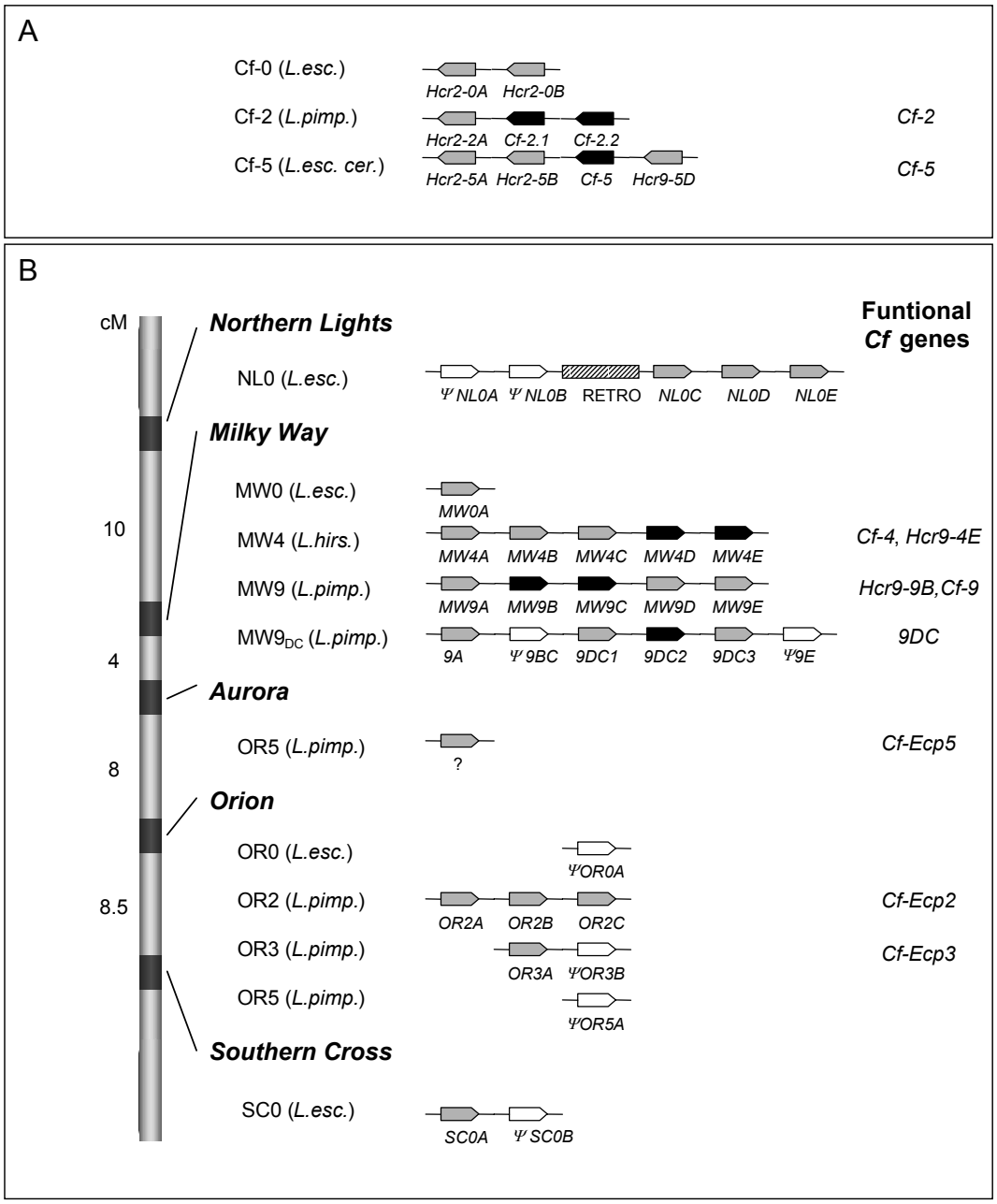

Figure 2. (**A**) Physical structure of *Hcr2* gene clusters on Chromosome 6 of different *Lycopersicon* haplotypes. Cf-0: *L. esculentum*; Cf-2: *L. pimpinellifolium*; Cf-5: *L. esculentum* var. *cerasiforme*. (**B**) Map position, genetic distance (cM) and physical structure of the *Northern Lights*, *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 five *Hcr9* loci relative to each other. In the middle, the physical organisation of each *Hcr9* cluster is shown. On the right, the functional *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.

C. fulvum resistance genes (Figure 2b). The *Hcr9-4D* homologue is the *Cf-4* gene which 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*., 1998b; 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). Furthermore, a patchwork of sequence similarities has been revealed for the *Hcr9* family members, strongly suggesting for 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. Polymorphisms 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 recombination alters the *Hcr9* number and composition of the clusters, and therefore leads 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 most distal *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 (Van der Hoorn *et al*., 2001a). 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 suggests that *9DC* is ancestral to *Cf-9* (Van der Hoorn *et al*., 2001a). Recently, the complete *9DC* cluster was isolated from *L. pimpinellifolium* LA1301, and a testcross positioned it at the *MW* locus (Figure 2b) (Kruijt *et al*., unpublished data). 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 (Van der Hoorn *et al*., 2001a). The recombination event that created the first *9DC* gene would have generated two identical intergenic regions, which likely increased the probability of misparing 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 (Kruijt *et al*., unpublished data). All three *9DC* genes confer responsiveness to Avr9 in tobacco under the 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 (Kruijt *et al*., unpublished data).

In a genus-wide screen for functional homologues of *Cf-4* and *Cf-9*, comprising eight wild tomato species, many Avr4- and Avr9-responsive tomato species were identified in different species (Kruijt *et al*., unpublished data). Avr4 recognition was observed in all eight wild species, except *L. pimpinellifolium*, whereas Avr9 recognition was present 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*., 2001b; 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 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 (Kruijt *et al*., unpublished data; R. Laugé, *pers. comm.*). It further suggested that *C. fulvum* is an ancient pathogen of the *Lycopersicon* genus, and has exerted a selection pressure for maintaining functional *Cf* genes.

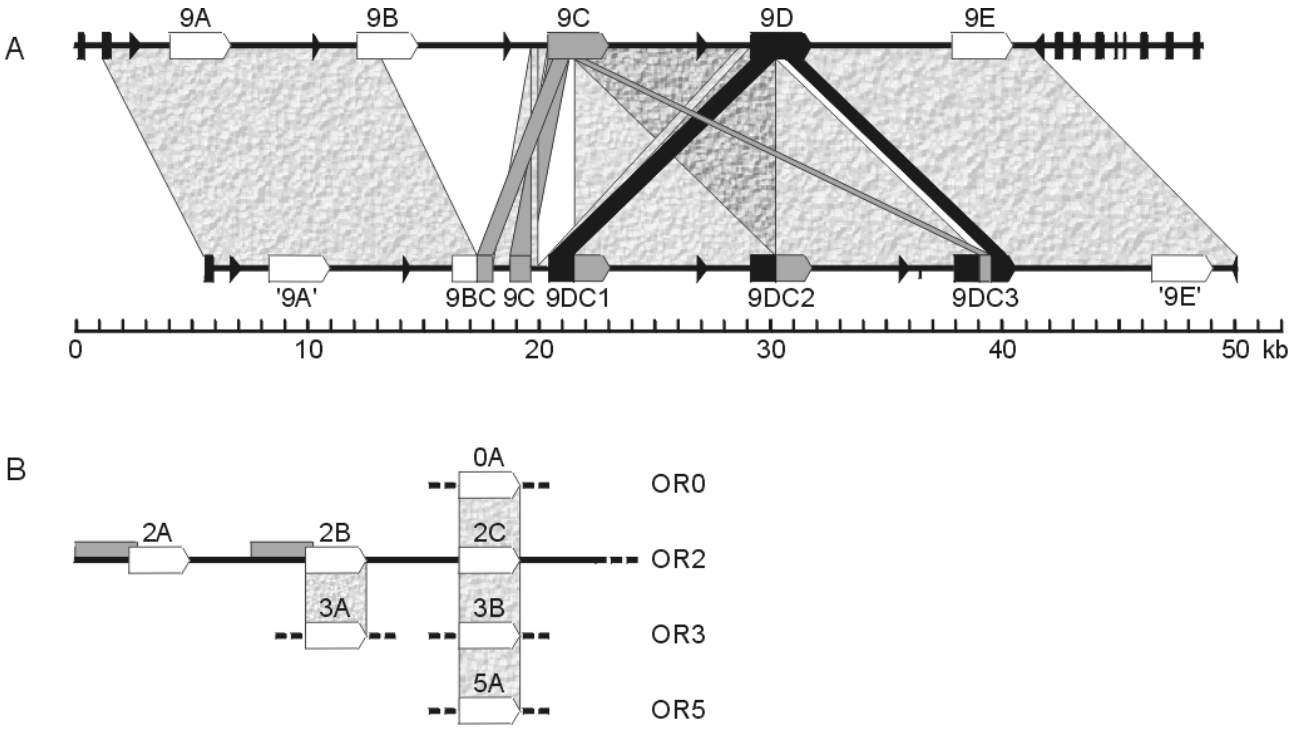

Figure 3. Schematic representation of the relationships between the *Cf-9* and *9DC* clusters, and between the *Orion Hcr9*s. **(A)** Relationships between the *Cf-9* (top) and *9DC* (bottom) clusters. Arrowed boxes represent complete *Hcr9*s; *Cf-9* (*9C*)-like sequences are depicted in grey; *Hcr9-9D* (*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 Kruijt *et al*., submitted. **(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*., CHAPTER 2.

Three *Cf-Ecp* genes were mapped and cosegregate with *Hcr9*s. The *Cf-Ecp2* and *Cf-Ecp3* resistance genes are located at the *Orion* (*OR*) locus (Haanstra *et al.,* 1999; Yuan *et al.,* 2002) whereas *Cf-Ecp5* is maps at the *Aurora* (*AU*) locus (Haanstra *et al.,* 2000a,b). The Cf-Ecp2 *OR* cluster contains three tandemly repeated *Hcr9s* (*OR2A*, *OR2B* and *OR2C*) (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*., CHAPTER 2). This duplication is probably the result of a recent intergenic unequal recombination. Orthologous *Hcr9s* were identified in different *Lycopersicon* species and haplotypes (Figure 2b) (De Kock *et al*., CHAPTER 2). The Cf-Ecp3 haplotype contains two *Hcr9*s (*OR3A* and Ψ*OR3B*) and the Cf-0 and Cf-Ecp5 *OR* loci both contain only one *Hcr9* (Ψ*OR0A* and Ψ*OR5A*, respectively). *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* elicitor Ecp2 and Ecp3, respectively, and trigger the HR-based resistance.

The orthologous genes of *OR2C* (Ψ*OR0A*, Ψ*OR3B* and Ψ*OR5A*) are almost identical, whereas also the other *OR Hcr9*s are very homologous (Figure 3b). Based on the shared polymorphic sites, the *OR Hcr9*s are subsequently most related to *ΨNL0A*, *ΨNL0B, NL0D* and *NL0E*. In contrast, the intergenic regions are very unique for the *OR* locus. The *MW* and *SC* clusters harbour several *LipoxygenaseC* (*LoxC*) exons that are thought to have coduplicated 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 Hcr9s* show 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.

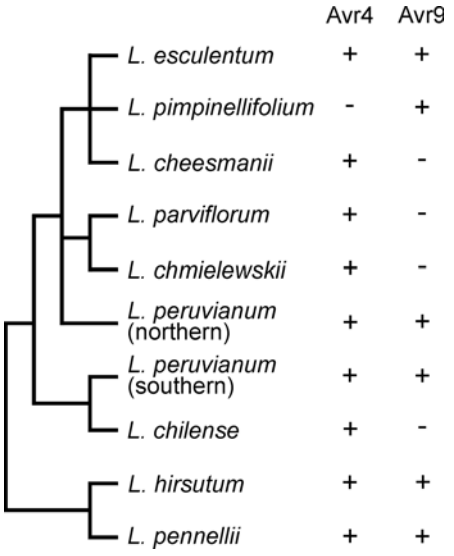

Figure 4. Tentative representation of phylogenetic relationships between the *Lycopersicon* species and Avr4 and Avr9 responsiveness within these species. Avr4 and Avr9 responsiveness was introduced in cultivated tomato (*L. esculentum*) from wild tomato species. This figure is adapted from Kruijt *et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

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. The major mechanism of generation of novel variation appears to be sequence exchange between homologues. Duplications, translocations, intra- and intergenic recombinations, gene-conversions and point mutations have all been reported. 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 exerted selection pressure for maintenance of these functional *Cf* genes in wild tomato populations.

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 unknown function, an extracellular LRR 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). 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 LRR copy number 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. Many 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 LRR copy number and add 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 located in the B-domain and in the putative solvent exposed sites 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*., 2001b, 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*., 2001b; 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).

An 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 LRRs of Hcr2s and Hcr9s interact with a shared component of the signal transduction cascade that initiates the defence responses, whereas the more variable N-terminal domains play a role in specific recognition (Dixon *et al.*, 1996, 1998). In contrast, amino-acid variation in the *OR* Hcr9s is dispersed over the protein, as here variation is also found in the C-terminal comprising LRRs 18-24, the loop-out, and the acidic E-domain. Based on these differences, the *OR* Hcr9s comprise a different class of Hcr9 proteins.

At the C-terminus of Hcr9s a dilysine motif for targeting to the endoplasmatic 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). However, 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*., 2001c). 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 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 rather than the rule (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 direct binding 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 analysis 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 autonecrosis, whereas the *L. pimpinellifolium* allele (*Rcr3*^{pim}) that was co-introgressed with *Cf-2* into cultivated tomato*,* can suppress this autonecrosis. 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, and it was suggested that this phenomenon resides at the level of perception of *C. fulvum* Avr factors, 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. A type of 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), that 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,b) 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 (M. Kruijt and R. Laugé, unpublished data) suggesting that the full signal transduction cascade required for Cf protein mediated signalling is conserved throughout the Solanaceae.

Signal transduction mediated by 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 resulted 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 (De Jong *et al.*, 2004). Phosphatidic acid could play a role in triggering responses such as an oxidative burst. 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 thio-redoxin (CITRX) was identified as a negative regulator of Hcr9s but not of Hcr2s (Rivas *et al*., 2004). Recently, gene-shuffling generated and natural *Hcr9*s were identified that induce a ligand-independent HR in tobacco species (Wulff and Kruijt, unpublished data). 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 protein mediated signalling. Several candidate genes have been 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).

Receptor like proteins involved in other pathosystems

Many genome and EST sequencing projects have recently been initiated and 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 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*., 2003; 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 accurately mapped to Chromosome 11 (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). However, successful complementation analysis with *Ve1* and *Ve2* in susceptible tomato has not been described yet. 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 both genes differ in the C-terminal part (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 structure of *Ve*1 and *Ve*2 comprises a signal peptide, 38 imperfect LRRs, a membrane-spanning hydrophobic sequence and a C-terminal with a cytoplasmic endocytosis signal (YXXΦ) (Kawchuk *et al*., 2001). The C-terminal domain of Ve2 contains additionally a PEST-like sequence, which is often involved in ubiquitisation, internalisation and degradation of proteins (Rogers *et al*., 1986). 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 lycopersicoides* (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 *Ve*1 in other solanaceous species may indicate once more 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, also in the absence of a pathogen and *HcrVf3* may be a pseudogene or may have a very low transcription level (Vinatzer *et al*., 2001). By transgenic complementation analysis in a susceptible apple cultivar, *HcrVf2* was recently proven 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. It was further shown that two sequential duplication events may 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. Therefore, isolation of additional *HcrVf* loci would shed more light on *HcrVf* gene evolution and could reveal the existence of sequence exchange between homologues as observed in the *Hcr9* gene family.

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 in which domains B and the N-terminal part of domain C are 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. 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, however, was identified in *Arabidopsis* accession Ler-0, which appears to encode for an RLP (Tör *et al*., 2004). This *Cf*-like gene was used to complement susceptible Col-*rpp27* plants. Progeny from these plants appeared resistant to at least five Col-compatible *P. parasitica* isolates suggesting that the RPP27 protein mediates resistance against *P. parasicita*. Surprisingly, these results indicate that resistance against *P. parasitica* can be 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 are 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. Similar 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), also proving that EIX must 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), which is lifted upon EIX treatment by translocation of proteins involved in this 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 protein may induce receptor-mediated endocytosis, thus allowing the receptor and/or EIX to interact with the 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). This is 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 *Cf*-like genes confer resistance to pathogens that grow extracellularly. The proposed extracellular LRRs and plasma-membrane localisation of Cf-like 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, biochemical data on elicitor perception is still limited. Besides the PEST- and endocytosis signals present in some RLPs, these receptor-like proteins lack distinct signalling domains like kinase motifs. Several downstream signal transduction components

have been identified, butit 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 can 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).

No data on the matching elicitors of the *Cf*-like genes *Ve*, *HcrVf2*, *RPP27* and their mode of action have been presented 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). The distribution is similar tot the *Cf* genes in tomato, with several complex loci containing three or more RLPs and others that are singlets or doublets (Törr *et al*., 2004). 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 to be implicated in disease resistance in Arabidopsis, enabling the deployment of Arabidopsis techniques to investigate the mechanisms of eLLR-TM function.

A challenge for the near future is to investigate whether RLPs are mainly involved in recognising and rejecting pathogens or, additionally, play an important role in developmental processes. Most *Cf*-like genes are located in clusters and are highly conserved, yet each *Cf*-like gene appears to mediate recognition of only a single distinct elicitor. Detailed evolutionary studies have mainly focussed on the tomato *Cf* genes, and future studies on evolution of other *Cf*-like resistance genes will reveal whether they have evolved by similar mechanisms. Identification and functional analysis of novel *Cf*-like 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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Recognition of the *Cladosporium fulvum* Ecp2 elicitor in tomato and non-host plants

Resistance against the tomato fungal pathogen *Cladosporium fulvum* is often conferred by *Hcr9* genes (Homologues of the *C. fulvum* resistance gene *Cf-9*) that are located in the *Milky Way* cluster on the short arm of Chromosome 1. These *Hcr9* genes mediate recognition of matching fungal avirulence gene products. In contrast, the resistance gene *Cf-Ecp2* mediates recognition of the pathogenicity factor Ecp2 and is located in the *Orion* (*OR*) cluster on the short arm of Chromosome 1. The main part of this thesis concentrates on the cloning of the *Cf-Ecp2 Orion* (*OR*) cluster and the identification of the functional *Cf-Ecp2* resistance gene that mediates HR-mediated resistance upon Ecp2 recognition.

In CHAPTER 2, we report the map- and homology-based cloning of the *OR Hcr9* cluster. A method was optimised to generate clone-specific fingerprint data that were subsequently used in the efficient establishment of genomic DNA contigs. Three *Hcr9*s were identified as candidate *Cf-Ecp2* genes. By PCR-based cloning using specific *OR* sequences, orthologous *Hcr9* genes were identified from different *Lycopersicon* species and haplotypes. The *OR Hcr9s* are very homologous to each other. However, based on a relative low sequence homology to other *Hcr9*s, the *OR Hcr9*s are classified as a new subgroup. As a consequence, the origin and the mode of action of this unique class of *Hcr9*s may differ from the other *Hcr9*s. To support allele mining, mapping, cloning and mRNA profiling of tomato *Hcr9* genes, a resistance gene analogue (RGA) fingerprint method was developed to generate novel *Hcr9*-specific markers (CHAPTER 3). The presence of both conserved and variable sequence domains in *Hcr9*s is exploited using a combination of PCR amplification and subsequent digestion. By the development of a fluorescent end-labelling method for restriction fragments, referred to as A/T labelling, high-resolution size-separation and detection of the complex RGA fingerprint pattern with a LICOR automated sequencer became possible. The RGA fingerprint method was validated by the analysis of near-isogenic lines and the analysis of two *Orion* (*OR*) *Hcr9* loci harbouring the *Cf-Ecp2* resistance gene or the recessive *cf-ecp2* allele. We identified several RGA-markers cosegregating with *Cf-Ecp2* resistance that corresponded to the three *Hcr9*s that are located at the *OR* locus. In addition, results indicate that the *Hcr9* RGA fingerprint method facilitates the discrimination of highly homologous genes in the analysis of a mapping population. Finally, the *Hcr9* RGA fingerprint method was applied to study the *Hcr9* gene expression and showed that two out of the three *OR Hcr9*s were expressed *in planta*. The various methods to identify the functional *Cf-Ecp2* gene are described in CHAPTER 4. Transient expression in *Nicotiana* species and complementation analysis in tomato were exploited to test candidate *Cf-Ecp2* genes for the ability to mediate

Ecp2 recognition. Despite applying all commonly used functional assays, we were not able to identify which of the three *OR Hcr9* represents the functional *Cf-Ecp2* gene. Based on these results we have to conclude that recognition of the *C. fulvum* Ecp2 elicitor is not solely mediated by an *OR-Hcr9* and an additional tomato-derived HR-stimulating factor is required for *Cf-Ecp2*/*Ecp2* mediated resistance.

In addition to the three independent chapters on the cloning and identification of *Cf-Ecp2*, two related research topics were investigated. *Cladosporium fulvum* is a fungal pathogen of tomato that grows exclusively in the intercellular spaces of leaves. In tomato, recognition of elicitors is followed by a hypersensitive response (HR) resulting in resistance. However, HR-associated recognition of Ecp2 has also been observed in *Nicotiana paniculata*, *N. sylvestris*, *N. tabacum* and *N. undulata* that are non-host plants of *C. fulvum* (CHAPTER 5). Absence of Ecp2-recognition did not lead to growth of *C. fulvum* on *Nicotiana* plants. We show that HR-associated recognition of Ecp2 is mediated by a single dominant gene in *N. paniculata*. However, based on PCR- and hybridisation analysis this gene is not homologous to known *Cf*-genes.

DNA sequence analysis of the Avr proteins (so-called race-specific elicitors) Avr2, Avr4, Avr4E and Avr9 have revealed that the change from avirulence to virulence is associated with DNA mutations in Avr coding regions. The high frequency of these mutations are most likely the result of high selection pressure caused by the frequent use of matching *Cf* resistance genes in commercial tomato lines. However, the *Cf-Ecp* resistance genes have rarely been employed and previous research showed that no variation was found the Ecp elicitors. In CHAPTER 6 the Ecotilling method was used in strains of *C. fulvum* that have been collected world-wide to compare the sequence variation in Avr and Ecp elicitor encoding genes while the variation in ribosomal internal transcribed spacers (ITS) was used as evolutionary clock. No polymorphisms in ITS sequences were observed. Silent mutations in *Avr*s occurred more frequently. However, the very fast majority of the mutations in Avr proteins were associated with virulence and indicate a high selection pressure in *C. fulvum Avr* elicitor genes. In *Ecp* elicitor genes, however, mutations occurred rarely and were not associated with virulence. These results show a very high mutation rate in elicitor proteins and confirms the lack of selection pressure on the *Ecp* genes by *Cf-Ecp* resistance genes.

The thesis is concluded with a general discussion on Cf- and Cf-like proteins involved in disease resistance (CHAPTER 7). Current knowledge on the genetics and evolution of *Cf* genes, Cf protein characteristics, elicitor perception and signal transduction in the tomato - *C. fulvum* pathosystem is discussed. In addition, the current knowledge on *Cf*-like proteins that are involved in other pathosystems is presented. Finally, this chapter describes some future directions in research on the described pathosystems.

Herkenning van de elicitor Ecp2 van *Cladosporium fulvum* in tomaat en niet-waard planten

Pathogenen beschikken over verschillende strategieën om planten te kunnen infecteren. Planten hebben daarentegen verschillende barrières ontwikkeld om pathogenen tegen te houden of actief te bestrijden (HOOFDSTUK 1). Bij één van deze barrières zijn resistentie (*R*) genen betrokken. *R* genen stellen de plant in staat ziekteverwekkers te herkennen wanneer deze complementaire avirulentie (*Avr*) genen of elicitors bevatten. De interactie tussen de pathogene schimmel *Cladosporium fulvum* en zijn gastheer tomaat (*Lycopersicon*) is een geschikt modelsysteem om interacties tussen planten en ziekteverwekkers te bestuderen. Bij de resistentie van tomaat tegen *C. fulvum* zijn vaak *Cf* of *Hcr9* (homoloog van het *Cladosporium fulvum* resistentie gen *Cf-9*) resistentiegenen betrokken die genetisch gelokaliseerd zijn in het *Milky Way* cluster op de korte arm van Chromosoom 1. De *Cf* genen coderen voor eiwitten die waarschijnlijk in het plasmamembraan van de plantencellen verankerd liggen. Hierbij ligt het grootste deel van de Cf eiwitten, die rijk zijn aan leucine-rijke repeats (LRRs), aan de buitenkant van de cel. De Cf eiwitten zijn betrokken bij de specifieke herkenning van bepaalde avirulentie (Avr) eiwitten van *C. fulvum*. Deze Avr producten zijn echter niet essentieel voor de pathogeniteit van *C. fulvum.* De schimmel kan de *Avr* genen veranderen waardoor er geen herkenning meer plaats vindt en resistentie wordt doorbroken. Het resistentiegen *Cf-Ecp2* is gelokaliseerd in het *Orion* cluster op de korte arm van Chromosoom 1 en is betrokken bij de specifieke herkenning van de elicitor Ecp2. Er zijn aanwijzingen dat Ecp2 belangrijk is voor de pathogeniteit van *C. fulvum*. Het zal daarom moeilijk zijn Ecp2 te veranderen zodat het niet meer door de plant herkend kan worden, maar nog steeds de biologische functie behoudt. Er wordt daarom verwacht dat het *Cf-Ecp2* resistentiegen een bijdrage zal kunnen leveren aan duurzame resistentie. Het grootste gedeelte van dit proefschrift beschrijft het kloneren van het *Orion* cluster en de identificatie van het *Cf-Ecp2* resistentiegen. Tevens worden de herkenning van Ecp2 door verschillende *Nicotiana* soorten en de wereldwijde variatie van elicitors van *C. fulvum* beschreven in dit proefschrift. De resultaten vormen een wetenschappelijke basis voor de co-evolutie tussen planten en pathogenen en maken een voorzichtige voorspelling van de duurzaamheid van resistentiegenen mogelijk.

In het eerste deel van de *Cf-Ecp2* trilogie wordt het kloneren van het *Orion* cluster beschreven (HOOFDSTUK 2). De kloneringsstrategie is gebaseerd op het gebruik van gekoppelde moleculaire merkers en homologie met bekende *Cf* genen. Er is een methode geoptimaliseerd waarmee kloon-specifieke fingerprint gegevens gegenereerd zijn die

gebruikt werden voor het berekenen van genomische DNA contigs. In het *Orion* cluster zijn drie *Hcr9* genen geïdentificeerd die kandidaat zijn voor het *Cf-Ecp2* resistentiegen. Met behulp van een PCR-kloneringsstrategie die gebaseerd is op *Orion*-specifieke DNA sequenties zijn orthologe *Hcr9* genen geïdentificeerd in andere *Lycopersicon* haplotypen en soorten. De orthologe *Orion Hcr9* genen zijn onderling zeer homoloog. Echter, op basis van DNA- en eiwit homologie vormen ze een subgroep ten opzichte van de reeds bekende *Hcr9* genen. Dit geeft aanleiding tot een discussie over de evolutionaire afkomst van deze genen.

Een *Hcr9 resistance gene analogue* (RGA) fingerprint methode is ontwikkeld ter ondersteuning van het haplotyperen, kloneren en bestuderen van *Hcr9* gen-expressie (HOOFDSTUK 3). Deze methode genereert een nieuw type genetische merkers specifiek voor de genen waaraan het onderzoek wordt verricht. De aanwezigheid van zowel geconserveerde als zeer variabele DNA-sequentiedomeinen wordt gebruikt in een PCR-reactie die gevolgd wordt door een DNA-restrictie proces. De ontwikkeling van een fluorescente eind-labelingsmethode (A/T labeling) maakt het mogelijk het complexe RGA fingerprintpatroon met hoge resolutie te scheiden en te detecteren met een geautomatiseerde LICOR sequencer. De betrouwbaarheid van de RGA-fingerprint methode is getest met de analyse van bijna-isogene veredelingslijnen en de analyse van het dominante *Cf-Ecp2* en het recessieve *cf-ecp2* allel. Er zijn meerdere RGA-merkers geïdentificeerd die gekoppeld zijn aan *Cf-Ecp2* resistentie. Allen zijn afkomstig van de *Hcr9* genen die in het *Cf-Ecp2 Orion* cluster aanwezig zijn. Op basis van de verkregen resultaten kan geconcludeerd worden dat deze methode zeer geschikt is om gerelateerde genen van elkaar te kunnen onderscheiden. De methode is daarom tevens gebruikt bij het bestuderen van de expressie van *Hcr9* genen. Er is aangetoond dat twee van de drie *Hcr9* genen in het *Orion* cluster daadwerkelijk in de plant tot expressie komen.

Transiënte expressie in *Nicotiana* soorten en complementatie-analyse in tomaat is toegepast om de betrokkenheid van de drie *Cf-Ecp2* kandidaatgenen bij de herkenning van Ecp2 te analyseren (HOOFDSTUK 4). Ondanks het feit dat alle gangbare functionele methoden zijn gebruikt, is het niet mogelijk gebleken om het *Cf-Ecp2* resistentiegen aan te tonen. Op basis van de verschillende resultaten moet geconcludeerd worden dat er naast één van de drie *Orion Hcr9* genen nog een andere factor betrokken is bij de herkenning van Ecp2 en de daaraan gekoppelde resistentie. Tevens is er waarschijnlijk een extra component aanwezig die de geïnduceerde resistentierespons versterkt.

C. fulvum is een pathogeen van tomaat dat uitsluitend in de intercellulaire ruimtes van bladeren groeit. Herkenning van elicitor eiwitten door de plant leidt tot een overgevoeligheidsreactie en resistentie. Specifieke herkenning van Ecp2 wordt ook gevonden in *Nicotiana paniculata*, *N. sylvestris*, *N. tabacum* en *N. undulata* (HOOFDSTUK 5). Deze planten zijn echter geen gastheer voor *C. fulvum*. De specifieke herkenning van

Ecp2 is daarom opmerkelijk. Afwezigheid van Ecp2 herkenning in *Nicotiana* soorten leidt niet tot infectiemogelijkheden voor *C. fulvum*. Er is aangetoond dat een enkelvoudig dominant gen betrokken is bij de specifieke herkenning van Ecp2. Dit gen is echter niet verwant aan de resistentiegenen tegen *C. fulvum* van tomaat.

Uitgebreide analyse van Avr elicitoreiwitten van *C. fulvum* heeft in het verleden aangetoond dat het omzeilen van herkenning door de plant wordt veroorzaakt door DNA mutaties in de *Avr* elicitorgenen. De hoge frequentie van mutaties is hoogst waarschijnlijk veroorzaakt door een hoge selectiedruk als gevolg van het gebruik van corresponderende resistentiegenen in de commerciële tomatenveredeling. De *Cf-Ecp* resistentiegenen zijn daarentegen niet of nauwelijks gebruikt in de veredeling en onderzoek heeft in het verleden aangetoond dat er weinig of geen variatie aanwezig is in de corresponderende *Ecp* elicitorgenen. De *EcoTILLING* mutatiedetectie-methode is gebruikt om de sequentievariatie in de geconserveerde ribosomale genen en variabele tussenliggende gebieden te vergelijken met de variatie die aanwezig is in *Avr* en *Ecp* elicitors in isolaten van *C. fulvum* uit een wereldwijde collectie. De ribosomale genen en de tussenliggende gebieden zijn volledig geconserveerd. Niet-effectieve mutaties komen soms voor in *Avr* genen. Mutaties die geassocieerd zijn met het omzeilen van herkenning door de plant, wat resulteert in het doorbreken van resistentie, komen daarentegen veelvuldig voor in *Avr* genen. In *Ecp* genen komen mutaties ook voor, echter veel minder vaak dan bij de Avr elicitors. Deze mutaties hebben geen effect op het doorbreken van resistentie. De resultaten tonen een zeer hoge mutatiefrequentie aan in elicitoreiwitten en bevestigen dat er op de *Ecp* genen geen of weinig selectiedruk is als gevolg van het kleinschalige gebruik van *Cf-Ecp* resistentiegenen in de tomatenveredeling.

Het proefschrift wordt afgesloten met een algemene discussie over de functie van Cf en Cf-achtige eiwitten die betrokken zijn bij ziekteresistentie (HOOFDSTUK 7). Huidige kennis over de genetica, evolutie, elicitorherkenning en signaaltransductie processen in de tomaat - *C. fulvum* interactie wordt beschreven. Daarnaast wordt een overzicht gegeven van de betrokkenheid van Cf-achtige eiwitten bij andere plant-pathogeen interacties.

Nawoord

Ik voel me ozo heppie,
zo heppie deze dag
en als je vraagt: wat heppie
als ik eens vragen mag,
dan zeg ik: hoe wat heppie,
wat heppik aan die vraag,
heppie nooit dat heppieje
dat ik hep vandaag?

Gedicht Joke van Leeuwen

Het proefschrift is klaar...., dat geeft mij een *heppie* gevoel! Hiermee komt er een einde aan een voor mij belangrijke en leerzame periode. De afgelopen vijf jaar heb ik met veel plezier onderzoek verricht aan een onderwerp waarvan de details voor velen (misschien nog steeds?) als *aboekadabra* in de oren klonken. Bij aanvang van het project waren de verwachtingen erg hoog, maar al snel bleek dat verwachtingen en realiteit niet hand-in-hand hoeven te gaan. Ondanks dat ik het kloneren van *Cf-Ecp2* niet heb kunnen afronden, ben ik trots op hetgeen we wèl hebben weten te bereiken. Dit *boekje* mag dan wel mijn proefschrift zijn, velen hebben er direct of indirect hun steentje aan bijgedragen.

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Curriculum vitae

Maarten Johannes David de Kock werd op 4 augustus 1974 geboren in Ede. In juni 1992 behaalde hij aan het Christelijk Lyceum te Veenendaal het H.A.V.O. diploma. In datzelfde jaar begon hij zijn studie Laboratoriumtechniek aan de Internationaal Agrarische Hogeschool Larenstein te Velp, met als specialisatierichting Plantenbiotechnologie. Zijn stages heeft hij verricht bij de vakgroep Plantencytologie en -morfologie van de Wageningen Universiteit en bij de vakgroep Oecologie van de Katholieke Universiteit Nijmegen. In juni 1996 behaalde hij zijn diploma en in hetzelfde jaar begon hij een studie Plantenveredeling en Gewasbescherming aan de Wageningen Universiteit met als specialisatie Moleculaire Biologie. Bij het laboratorium voor Fytopathologie heeft hij als student mutatieonderzoek verricht aan elicitors van de schimmel *Cladosporium fulvum* die essentieel zijn voor de interactie van de schimmel met tomaat. In januari 1999 behaalde hij *cum laude* zijn doctoraal diploma. Hij vervolgde zijn werkzaamheden bij het Laboratorium voor Fytopathologie als junior-onderzoeker. In november van datzelfde jaar begon hij zijn promotieonderzoek bij het Laboratorium voor Plantenveredeling en het Laboratorium voor Fytopathologie aan de Wageningen Universiteit. De resultaten van dit door STW gefinancierde project staan beschreven in dit proefschrift. Sinds 1 augustus 2004 heeft hij een aanstelling als wetenschappelijk onderzoeker bij het Laboratorium voor Fytopathologie. Zijn onderzoeksproject richt zich op de productie van biosurfactants en de betrokkenheid van deze stoffen bij de beheersing van oömycete pathogenen.

Bibliographic abstract

Cladosporium fulvum is a fungal pathogen of its only host plant tomato. Resistance against *C. fulvum* is often conferred by *Hcr9* genes (homologues of the *C. fulvum* resistance gene *Cf-9*) that are located at the *Milky Way* cluster on the short arm of Chromosome 1. *Hcr9* resistance genes mediate the recognition of fungal avirulence or elicitor proteins. Recognition is subsequently followed by a hypersensitive response (HR) resulting in resistance. Ecp2 is one of the fungal avirulence proteins and is specifically recognised by tomato plants containing the resistance gene *Cf-Ecp2* that is located at the *Orion* cluster on the short arm of Chromosome 1. The *Cf-Ecp2 Orion Hcr9* cluster and orthologous *Orion Hcr9*s of other tomato genotypes were cloned by a map-based, and homology-based cloning approach. A resistance gene analogue fingerprint method was developed that facilitated the mapping, cloning and mRNA profiling of *Hcr9* genes. The *Orion Hcr9*s are very homologous. However, based on the relative low sequence homology to other *Hcr9*s, the *Orion Hcr9*s are classified as a new subgroup. Transient expression experiments and complementation analysis were not successful for the identification of the *Cf-Ecp2* gene that mediates Ecp2 recognition. It was concluded that Ecp2-recognition is not solely mediated by one of the *Cf-Ecp2 Orion Hcr9*s. HR-associated recognition of Ecp2 has additionally been observed in *Nicotiana paniculata*, *N. sylvestris*, *N. tabacum* and *N. undulata* that are non-host plants of *C. fulvum*. Absence of Ecp2-recognition did not lead to growth of *C. fulvum* on *Nicotiana* plants. HR-associated recognition of Ecp2 is mediated by a single dominant gene in *N. paniculata*. However, this gene is not homologous to known *Cf*-genes. Resistance in tomato is broken by *C. fulvum*. Change from avirulence to virulence of *C. fulvum* is associated with DNA mutations in the elicitor protein coding regions that affect the recognition by tomato. The Ecotilling method was used to investigate the sequence variation in Avr and Ecp elicitor encoding genes. The very fast majority of mutations in Avr proteins were associated with virulence and indicate a high selection pressure in *C. fulvum Avr* elicitor genes. In *Ecp* elicitor genes, however, mutations occurred rarely and were not associated with virulence. The results show an unusual high mutation rate in elicitor proteins and confirms the lack of selection pressure on the *Ecp* genes by *Cf-Ecp* resistance genes.

The training and supervision plan was completed at the Graduate School Experimental Plant Sciences (EPS), Wageningen University.

1. Participation in postgraduate courses and workshops:
   a) Signalling in plant development and defence.
   b) R&D Management in AGRO-life Sciences.
   c) Interactions between Plants and Attacking Organismes.
   d) Bioinformatics.
   e) Disease resistance in plant.

2. Oral and poster presentations at international conferences:
   a) International Congress of Plant Molecular Biology, Quebec, Canada. Poster: Mapping and cloning of the tomato resistance gene *Cf-ECP2*, targeted against a pathogenicity factor of *Cladosporium fulvum* (2000).
   b) Symposium Durable Disease Resistance, Ede, The Netherlands. Oral presentation: Resistance genes targeted against a pathogenicity factor are more durable (2000).
   c) International Congress on Molecular Plant-Microbe Interactions, Madison, USA. Poster: An efficient map-based cloning strategy of plant gene clusters: the tomato *Cf-ECP2* gene as example (2001).
   d) Plant and Animal Genome Conference XI, San Diego, USA. Poster: Mapping and cloning of tomato resistance gene *Cf-ECP2*, targeted against a virulence factor of *Cladosporium fulvum* (2003).

3. Oral and poster presentations at annual meetings:
   a) EPS theme meetings (2000 - 2002).
   b) Annual EPS PhD students day (2000 - 2003).
   c) Annual ALW meetings (2000 - 2004).

4. Participation in organisation:
   a) Member EPS Education Committee (December 2000 - November 2003).

The work described in this thesis was carried out in the Graduate School Experimental Plant Sciences at the Laboratory of Plant Breeding and the Laboratory of Phytopathology, Wageningen University. Main financial support of the research project was provided as STW project "Isolation and exploitation of resistance genes in Solanaceous species of which some are targeted against fungal pathogenicity factors" (WGC.5107). The J.E. Jurriaanse Stichting financially supported the reproduction of this thesis.

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