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THE TOMATO–*CLADOSPORIUM FULVUM* INTERACTION: A Versatile Experimental System to Study Plant-Pathogen Interactions

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■ **Abstract** Over the past 20 years, the interaction between the biotrophic fungal pathogen *Cladosporium fulvum* and tomato has developed into a versatile experimental system for molecular plant pathology and resistance breeding. This interaction provided the resources for cloning of fungal avirulence genes for the first time and interesting clues on recognition of their extracellular products by tomato, as well as mechanisms employed by the fungus to circumvent this recognition. A wealth of information has become available on the structure and genomic organization of *Cf* resistance genes. The occurrence of many clustered *Cf* homologues allows the generation of new genes with additional recognitional specificities by reshuffling. It is anticipated that potentially all proteins secreted by *C. fulvum* are recognized by one or more individuals in a population of tomato genotypes, a hypothesis that has been experimentally confirmed. The future challenge will be to elucidate the mechanisms of perception of avirulence factors and the subsequent signaling eventually leading to activation of host defense responses.

INTRODUCTION

Cladosporium fulvum

Cladosporium fulvum (Cooke, syn. *Fulvia fulva*) belongs to the class of Fungi Imperfecti, also called Deuteromycetes. The fungus produces asexual conidia on single conidiophores and lacks fruiting bodies. A sexual stage has never been observed. Its morphology resembles that of filamentous Ascomycetes; mycelium is well-developed, septated, and branched, and the major polysaccharides of the cell walls of the hyphae consist of glucans and chitin.

C. fulvum was first described by Cooke in 1883 (14). It is a pathogenic fungus for which species of the genus *Lycopersicon* serve as a host and is thought

to originate from South America, the center of diversity of tomato. While most of the *Lycopersicon* species that occur in South America are resistant, cultivated tomato (*Lycopersicon esculentum* Mill.) is susceptible. The fungus primarily is a pathogen of leaves, causing a leaf mould, but it is known to attack stems, flowers, and fruits as well (9). *C. fulvum* usually affects greenhouse-grown tomato plants and is less frequently found on outdoor crops. The fungus probably overwinters in the greenhouse as conidia that remain on infected foliage (9). Today, however, tomatoes are grown year-round, making survival of the fungus less critical.

Polymorphism within the *C. fulvum* population as revealed by AFLP analysis (109) is lower than 5% (Figure 1). Analysis of several isolates collected from different parts of the world shows genetic clustering of strains originating from the same geographical region. These observations, added to the fact that a sexual

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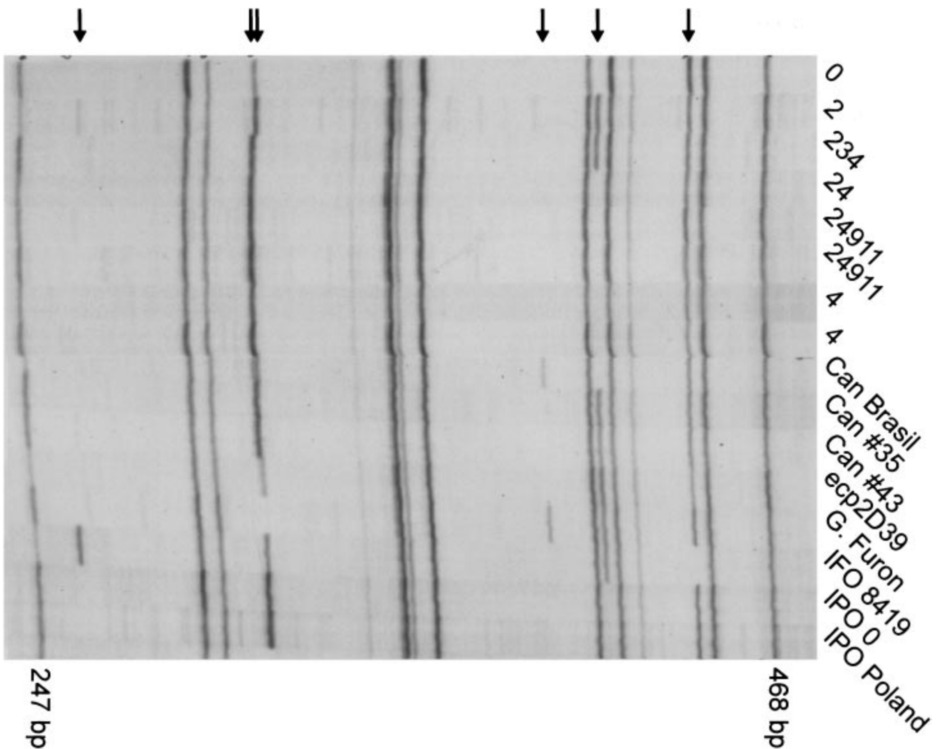

Figure 1 AFLP DNA fingerprints of various strains of *C. fulvum* collected throughout the world. Genomic DNA was digested with *Eco*RI and *Mse*I, adapters specific for these restriction enzymes were ligated, and selective PCR was performed using +2 (EcoAA) and +1 (MseA) primers (109). The six polymorphisms present on this part of the exposed gel, showing some of the strains that were analyzed, are indicated by arrows on the top.

cycle has never been observed for this fungus, suggest that the *C. fulvum* population consists of a single clonal lineage (P Vossen & T van der Lee, unpublished data).

The Compatible Interaction between Tomato and *C. fulvum*

Inoculation of conidia from a virulent strain of *C. fulvum* onto a susceptible tomato genotype results in a compatible interaction. At high relative humidity conidia germinate on the leaf surface and form thin "runner hyphae" that grow over the leaf surface (6, 20, 67). Penetration of stomata, which are abundantly present at the lower side of leaves, occurs at random. Hyphae that have entered the leaf subsequently increase in diameter and extensively colonize intercellular spaces between spongy mesophyll cells. During colonization, the hyphae are closely appressed to mesophyll cells, causing slight indentation (20, 68). As a carbon source, a wall-bound invertase of the fungus hydrolyses apoplastic sucrose into glucose and fructose, which are taken up and subsequently converted into mannitol by a fungal mannitol dehydrogenase. Since mannitol cannot be metabolized by the plant, it allows retention of carbon solely for use by the fungus (52, 77). The major part of fungal biomass is concentrated around the vascular tissue (103, 117). This is probably due to the relatively high concentration of sucrose near transfer cells of the phloem. Eight to ten days after inoculation stromal aggregations are formed in substomatal cavities from which aerial mycelium is initiated. Unbranched conidiophores are produced on the lower side of the leaves. Although host cells do not appear to undergo significant structural changes, degeneration of host cells becomes apparent at times when sporulation is extensive.

The Incompatible Interaction between Tomato and *C. fulvum*

Inoculation of conidia of an avirulent strain of *C. fulvum* onto a resistant tomato genotype results in an incompatible interaction. During the early stages of infection, incompatible and compatible interactions are very similar. Conidia germinate, form "runner hyphae" and penetrate stomata. The striking difference from the compatible interaction is that infecting hyphae that come into contact with mesophyll cells induce various fast host responses. Cell wall appositions are formed (20, 42, 67) and callose deposition takes place (20, 67). Furthermore, there is an accumulation of phytoalexins (26) and pathogenesis-related (PR) proteins, such as P14 (28), which belongs to a family of proteins with unknown function, and 1,3-ß-glucanases and chitinases (51), which are hydrolytic enzymes that can degrade hyphal walls (5). However, the most typical phenomenon of the incompatible interaction is the hypersensitive response (HR). Mesophyll cells adjacent to the intercellular hyphae collapse, turn brown, and their cytoplasm becomes highly disorganized (20, 67, 68). Within the host cells, organelles degenerate, the endoplasmic reticulum (ER) swells, and the plasmalemma and tonoplast become disrupted. Fungal hyphae often appear swollen and curled, while those that are in contact with mesophyll cells collapse and show deposits of amorphous material at their outer surface (20, 67, 68).

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Pathogenicity and Virulence of *C. fulvum*

C. fulvum does not form specialized feeding structures such as haustoria to establish a compatible relationship with the plant, but obtains nutrients through the enlarged intercellular hyphae that are in close contact with host cells (20, 67). Several proteins and peptides are secreted by the fungus into the intercellular space and might induce leakage of nutrients, such as sucrose, from the host cells into the apoplast. The observed indentation of the host cell walls that are adjacent to the fungal hyphae could result from the activity of particular cell wall–degrading enzymes secreted by the fungus, but we do not have experimental proof for this.

In order to cause disease on tomato, *C. fulvum* is expected to require a set of essential pathogenicity factors (1). Loss of one such factor will result in complete loss of disease-causing ability. In addition to pathogenicity factors, the fungus is thought to produce several virulence factors that are not essential for causing disease, but that can modulate symptoms and are necessary for full parasitic fitness. Loss of a virulence factor results in less severe colonization of tomato leaves (1).

Since colonization of tomato leaves by *C. fulvum* remains restricted to the apoplast, exchange of molecular signals between the fungus and the plant has to occur extracellularly. A way to explore only this area, thus excluding symplastic components, is to isolate apoplastic washing fluid (AF) from *C. fulvum*–infected leaves (27). Detailed analysis of the protein composition of AF isolated from colonized tomato leaves indicated that it contains several secreted fungal proteins (25, 50).

The ExtraCellular Proteins (ECPs) are secreted by all strains of the fungus. Two of these proteins, ECP1 and ECP2, have been purified (50, 117) and their encoding genes have been isolated (105). Expression studies revealed that both genes are highly transcribed upon colonization of tomato leaves, indicating that they might play a role in virulence of the fungus (117). Deletion of these two *Ecp* genes from the genome of *C. fulvum* did not result in a reduction of disease symptoms when assayed on axenically grown, susceptible two-week-old tomato seedlings (65, 73). However, when six-week-old tomato plants that are normally infected in nature were inoculated with the ECP-deficient strains, a clear phenotype was observed (65). Although both strains still caused disease symptoms, the ECP-deficient strains were blocked in later stages of their development. The ECP1-minus strain colonized leaves as efficiently as did the wild-type strain, but sporulated less abundantly, whereas the ECP2-deficient strain colonized leaves poorly and hardly sporulated. Thus, since deletion of the *Ecp* genes in *C. fulvum* results in a decrease of parasitic fitness, both ECPs can be regarded as virulence factors.

Not all proteins secreted by the fungus, however, function as virulence factors. Spanu (91) deleted the *HCf-1* gene, a member of a family consisting of at least six genes encoding so-called hydrophobins (111), from the genome of *C. fulvum*. He hypothesized that hydrophobins present in the hyphae could act as "stealth" determinants that mask the fungus from detection by the host, thereby

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avoiding the activation of host defense responses. Pathogenicity tests with the HCf-1-minus strain, however, did not reveal clear differences between this strain and the wild-type strain. Deletion of a second hydrophobin gene (*HCf-2*) also did not affect pathogenicity of the fungus (PD Spanu, unpublished data). Possibly other members of the hydrophobin family can complement the deficiency of this gene. Interestingly, ectopic integration of a truncated copy of the *HCf-1* gene in the genome of *C. fulvum* resulted in co-suppression of this hydrophobin gene, giving a hydrophilic phenotype (36). The silenced transformants are being tested for their pathogenicity on tomato, since the expression of additional *HCf*-homologues could also be reduced (PD Spanu, unpublished data).

Thus, to date no or only partial reduction of virulence has been observed upon deletion of candidate pathogenicity or virulence genes from the genome of *C. fulvum*. It appears that pathogenicity of the fungus relies on a set of virulence factors that are dispensable on their own, but that in combination allow the fungus to be fully virulent on tomato.

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RACE-SPECIFIC ELICITORS AS DETERMINANTS OF GENOTYPE-SPECIFIC AVIRULENCE

Tomato Genotype-Specific Resistance

The interaction between tomato and *C. fulvum* is a typical gene-for-gene relationship. This concept, which was independently proposed by Flor (34, 35) and Oort (78), states that for each dominant resistance (*R*) gene (in this case *Cf* gene) there is a matching dominant avirulence (*Avr*) gene present in the fungus. When both partners are present recognition occurs, leading to active resistance of the plant and avirulence of the fungus. If no matching *Cf* and *Avr* genes are present, recognition does not occur and the fungus successfully colonizes the plant (23, 24).

Many different *Cf* resistance genes are present in wild tomato species, such as *L. pimpinellifolium*, *L. chilense*, *L. hirsutum*, and *L. peruvianum*. In the past, monogenic resistance traits have been introgressed into commercially grown tomato varieties to protect them against the fungus (56, 93). These single resistance loci, however, were eventually overcome by new strains of *C. fulvum* (18, 44). Pyramiding of several resistance genes by plant breeders subsequently led to the appearance of more complex races of the fungus that are able to overcome various combinations of resistance genes. Many races of *C. fulvum* have been described (44, 70). For research purposes, resistance traits such as *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* have been introgressed into the tomato cultivar MoneyMaker (MM), which does not contain any known resistance gene against *C. fulvum* (7). Repeated backcrossing resulted in near-isogenic lines (NILs; "MM-Cf" plants) that allow studies on the physiological, biochemical, and molecular mechanisms of specific recognition of a pathogen by its host, based on the presence of single gene-for-gene pairs.

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In a search for avirulence factors that match particular resistance genes, De Wit & Spikman (27) showed that AF, which was obtained from compatible *C. fulvum*-tomato interactions, contains a set of race-specific fungal elicitors. In contrast to ECPs, these avirulence factors are produced only by particular strains. Injection of AF into a leaf of a resistant tomato genotype resulted in HR, which is visible as necrosis and/or chlorosis throughout the injected area of the leaf. Injection of the various NILs with AF resulted in differential induction of HR, allowing the identification of various avirulence factors.

Isolation and Characterization of the AVR4 and AVR9 Elicitors and Their Encoding Genes

The observation that injection of AF obtained from a compatible *C. fulvum*–tomato interaction specifically caused HR in resistant NILs was exploited to isolate elicitors that corresponded to the resistance genes *Cf-4* and *Cf-9*. Proteins present in AF were fractionated using various biochemical separation techniques, such as preparative native low pH PAGE, gel filtration, ion-exchange, and reversed-phase chromatography. The various fractions were analyzed for the presence of HR-inducing activity by injection into leaflets of MM-Cf4 or MM-Cf9 plants. This approach allowed identification of single proteins that induced HR in MM-Cf4 [AVR4 (49)] or MM-Cf9 [AVR9 (89)] plants. Amino acid sequence information was obtained for each elicitor protein and corresponding cDNA clones and genes were obtained by screening libraries of a compatible MM-Cf5/race 5 interaction with radiolabeled degenerate oligonucleotide probes (49, 104, 107). Both AVR4 and AVR9 elicitor proteins are encoded as pre-pro-proteins that contain a signal sequence for extracellular targeting. Upon secretion both proteins undergo further processing at the N terminus, while AVR4 undergoes additional processing at the C terminus. The mature AVR4 protein consists of 86 to 88 amino acids, depending on the degree of processing at the N or C terminus (53), whereas the mature AVR9 peptide consistently contains 28 amino acids (89). Both AVR4 and AVR9 peptides contain an even number of cysteine residues, eight in AVR4 and six in AVR9.

*Avr*4 lacks introns while the *Avr*9 gene contains one short intron of 59 base pairs in the open reading frame (ORF) (104) (49). Transfer of *Avr*4 or *Avr*9 into the genome of strains of *C. fulvum* that do not produce the AVR proteins made them avirulent on tomato genotypes that contain the corresponding *Cf* resistance genes (49, 104). This proved that the genes encode true avirulence factors that fit the gene-for-gene model. The reciprocal experiment was also performed with the *Avr*9 gene. Replacement of the *Avr*9 gene by a hygromycin resistance cassette in strains normally avirulent on the Cf9 tomato genotypes resulted in virulence on these plants (72; see below).

Structure-Function Analysis of AVR Proteins

The three-dimensional structure of the AVR9 peptide has been extensively studied. As the *Avr*9 gene is transcribed only in planta, the ORF of the *Avr*9 gene had to

be fused to a promoter that is constitutively active. In this way, production of the AVR9 peptide was obtained from in vitro grown fungus. Constitutive secretion of the AVR9 peptide was achieved by constructing a transgenic strain of *C. fulvum* that contained a fusion between the promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene of *Aspergillus nidulans* and the *Avr*9 ORF (106). In culture filtrates of the transgenic strain, a mixture of AVR9 elicitor peptides of 32, 33, and 34 amino acids in length is present. This suggests that production of the mature AVR9 peptide of 28 amino acids in planta is due to the additional activity of plant proteases.

The 33-amino acid AVR9 peptide was purified and ^{1}H-NMR analysis revealed that the peptide contains three antiparallel strands that form a compact region of ß-sheet and two solvent-exposed loops, a short one between Phe26 and Leu2g and an extended one ranging from Thr12 to Gln20 (108) (Figure 2). Exact determination of the molecular mass of the peptide by electrospray mass spectrometry revealed that all 6 cysteine residues are involved in disulfide bonds, as the mass was 6 Daltons lower than the calculated mass of the linear protein. The three disulfide bonds provide a rigid structure to the peptide, which makes it resistant to degradation by plant and fungal proteases present in the extracellular spaces. A comparison between AVR9 and other small, cysteine-rich peptides, such as serine protease inhibitors, ion channel blockers, and growth factors, revealed a strong structural relatedness (108, and references therein). All these proteins contain a so-called cystine knot; the disulfide bonds between Cys1-Cys4, and Cys2-Cys5, together with the intervening protein backbone, form a ring through which the third Cys3-Cys6 disulfide bond penetrates (79). Structurally, AVR9 is most related to carboxy peptidase inhibitor, of which the three-dimensional X-ray structure has been determined (83) (Figure 2).

To determine which of the amino acid residues within the AVR9 peptide are important for necrosis-inducing activity, a mutational approach was followed using the potato virus X (PVX) expression system (10). Recombinant PVX constructs were made that contain the ORF of *Avr*9, in which the codons for each of the 28 amino acids had been independently changed into a codon for alanine ("alanine scan"). The severity of the systemic necrosis caused by the PVX-produced mutant AVR9 peptides upon inoculation onto MM-Cf9 genotypes was compared with the activity of the wild-type PVX::*Avr*9 construct. In addition to this nonbiased approach, various surface-exposed residues were changed into homologous amino acid residues. It was concluded that the hydrophobic residues present in both solvent-exposed loop regions are essential for the necrosis-inducing activity of the AVR9 peptide (59). A PVX::[Phe21Ala]*Avr*9 derivative, for example, in which the hydrophobic phenylalanine residue on the surface-exposed side of AVR9 had been changed into an alanine residue, did not induce any necrosis on MM-Cf9 plants. Additional support for an important role of the hydrophobic region of the AVR9 peptide in elicitor activity was obtained by Mahé and coworkers (71), who chemically synthesized some crucial AVR9 mutant peptides. The [Phe21Ala]AVR9 peptide was still able to fold correctly, but did

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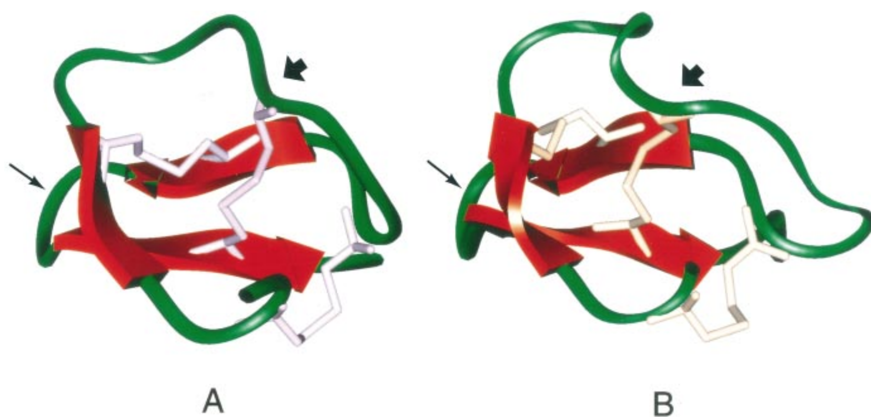

Figure 2 Three-dimensional structures of AVR9 (*A*) and carboxy peptidase inhibitor (*B*). The red strands form an antiparallel ß-sheet, and the two solvent-exposed loops are indicated in green. The thin arrow points to the short exposed loop, and the thick arrow indicates the extended loop. The three disulfide bridges between cysteine residues present in each molecule are indicated in pink.

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not show any necrosis-inducing activity upon injection into leaflets of MM-Cf9 plants.

A strictly positive correlation was found between necrosis-inducing activity of the AVR9 mutants and their affinity for polyclonal antibodies raised against the wild-type AVR9 peptide (71). This suggests that specific recognition of AVR9 by the plant depends on only one or more epitopes in the peptide that are identical to the one(s) that serve(s) as antigenic determinant(s) of the peptide in mammals (71). Possibly AVR9 contains only one strong antigenic determinant, of which Phe21 is one of the essential residues, as [Phe21Ala]AVR9 is not recognized by the antibodies.

Not much is known about the three-dimensional structure of the AVR4 protein. A hydrophobicity plot of AVR4 indicated that the protein has a hydrophobic center. In a peptide scan (PEPSCAN) analysis of the mature AVR4 protein, 77 overlapping peptides of 12 amino acids each were synthesized. Incubation of the individual peptides with polyclonal antibodies raised against AVR4 indicated that the hydrophobic central area of the protein is crucial for antigenicity. This suggests that, similar to AVR9, the AVR4 protein may have a hydrophobic, solvent-exposed face (W Schaaper & MHAJ Joosten, unpublished data). Accurate mass determination of the mature AVR4 protein showed that all eight cysteine residues of the protein are involved in disulfide bonds.

Circumvention of *Cf-4*- and *Cf-9*-Mediated Resistance by *C. fulvum*

Detailed analysis of naturally occurring strains of *C. fulvum* that are virulent on tomato genotypes containing the *Cf-4* or *Cf-9* resistance gene revealed that the fungus employs two distinct mechanisms to avoid recognition by the host plant. Strains that circumvent the *Cf-4* resistance gene were found to contain a single point mutation in the ORF of the *Avr*4 gene, resulting in "virulent" *avr*4 alleles. The mutant *avr*4 alleles, which have been found in strains from The Netherlands, Poland, France, and the USA, have been grouped into three classes (53). In one class, a single cysteine residue was substituted by a tyrosine residue. This change was found in Cys4, Cys5, or Cys8 (counting from the N terminus). In a second class, single amino acids between Cys4 and Cys5 were changed. In one case, a change from threonine to isoleucine and in another, a change from tyrosine to histidine was found. Expression of the virulent *avr*4 alleles in MM-Cf4 plants by PVX revealed that some of these alleles encode an AVR4 protein that is still recognized, because mild and delayed HR was observed in some cases (53). The fact that strains of *C. fulvum* that carry such an *avr*4 allele have become virulent on MM-Cf4 plants probably results from high instability of the encoded mutant protein (53). In a third class, a single nucleotide deletion had occurred, which resulted in a frameshift of the ORF of *Avr*4. In this mutant, only 13 N-terminal amino acids identical to the mature AVR4 protein remained. The absence of any functional AVR4 protein in this strain, yet with no loss of pathogenic fitness,

indicates that the AVR4 protein is probably dispensable for full virulence of the fungus (53).

Similarly, the AVR9 protein does not appear to function as a virulence factor for *C. fulvum*. *Avr*9 gene replacement did not affect the pathogenic fitness of the fungus (72). Furthermore, natural strains in which the entire *Avr*9 gene is absent are able to colonize leaves of MM-Cf9 tomato plants without a decrease in virulence (107). Karyotype analysis of natural strains of *C. fulvum* that circumvent *Cf-9*-mediated resistance revealed that lack of the gene could be the result of a large genomic deletion (98).

The identification of three different classes of point mutations, resulting in six different *avr*4 alleles, is surprising in light of the observation that the *C. fulvum* population consists of a single clonal lineage, as mentioned earlier. This suggests that circumvention of *Cf-4*-mediated resistance by *C. fulvum* is the result of several independent events. The extensive use of the *Cf-4* gene in commercial tomato crops has possibly exerted a strong selection pressure on this locus in different parts of the world.

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VIRULENCE FACTORS WITH ELICITOR PROPERTIES

Extracellular Protein ECP2 of *C. fulvum*

In contrast to AVR4 and AVR9, the ECP1 and ECP2 proteins are essential for full virulence of the fungus (65). Based on the lack of redundancy of these virulence factors, it is expected that tomato genotypes that respond with HR upon specific recognition of the ECP1 or ECP2 proteins carry resistance genes that are potentially durable.

Analysis of the two *Ecp* genes showed that disruption of *Ecp2* caused the most severe reduction in pathogenic fitness (65). Therefore, the virulence factor encoded by this gene was initially selected for further studies. To search for a tomato genotype that responds with HR upon exposure to ECP2, the ORF of the *Ecp*2 gene was inserted in the PVX expression vector and various tomato genotypes were inoculated with the recombinant virus (64). In total, 21 lines that had previously been selected for resistance against *C. fulvum* in early breeding programs were screened. Four lines, originating from the same *L. pimpinellifolium* ancestor, displayed systemic necrosis upon inoculation with PVX::*Ecp*2. Injection of purified ECP2 protein into these plants resulted in HR. Analysis of the offspring of a cross between the responding lines and MM-Cf0 plants revealed that recognition of ECP2 is based on the presence of a single dominant gene, designated *Cf-ECP2* (64). Significantly, all strains of *C. fulvum* analyzed so far produce the ECP2 protein and are avirulent on the four responding lines. However, strains in which the *Ecp*2 gene has been deleted become partially virulent on *Cf-ECP2* genotypes, thus proving that resistance of these plants is solely based on recognition of ECP2 (64). Since loss of the *Ecp*2 gene also results in a severe reduction of virulence, the *Cf-ECP2* gene is envisaged to be durable. This durability, however,

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can be of limited value if selection occurs on point mutations in the ORF of *Ecp*2 that affect recognition of the encoded protein and subsequent development of HR but have no effect on its function as virulence factor. Furthermore, loss of ECP2 could be compensated for by other factors. Introduction of the *Cf-ECP2* gene in widely used commercial tomato cultivars will reveal whether this gene provides long-lasting protection against *C. fulvum*.

As far as fungal pathogens are concerned, the avirulence factor NIP1 of *Rhynchosporium secalis*, the causal agent of barley leaf scald, also plays a role in virulence (112, 113). The protein acts as an aspecific toxin on all barley varieties, other cereals, and bean. Furthermore, it specifically induces active resistance in barley cultivars that contain the resistance gene *Rrs1* and, therefore, can be regarded as the product of avirulence gene *AvrRrs1* (84). The *Rrs1* gene is expected to be durable, as mutations in the *AvrRrs1* gene that allow *R. secalis* to circumvent *Rrs1*-mediated resistance result in a decrease of virulence.

The targeted search for durable resistance genes has been successful for the interaction between tomato and *C. fulvum*, but might also be feasible for other plant-pathogen interactions that fit the gene-for-gene model. However, for successful application of the identified *R* gene in molecular resistance breeding, a prerequisite is that HR-associated recognition of a virulence factor by the host should result in effective growth inhibition of the pathogen. This HR-based resistance is probably most effective against biotrophic or obligate fungal pathogens, such as *Phytophthora infestans* and *Oidium lycopersicum*.

Since PVX is also infectious on other solanaceous plants, such as *Nicotiana* species, the PVX::*Ecp*2 construct was also used to screen for HR-associated recognition of the ECP2 protein outside the limited host range of *C. fulvum*. Inoculation of 44 accessions belonging to wild species of the genus *Nicotiana* resulted in the identification of two accessions of *N. paniculata* in which all plants responded with HR to ECP2. Rather than developing a systemic HR, these plants showed small lesions only on the inoculated leaves without further spread of the virus (R Laugé, P Goodwin, PJGM De Wit & MHAJ Joosten, unpublished data). These symptoms are very similar to the resistance response observed in *Nicotiana* species that contain the *N* gene for resistance against tobacco mosaic virus (114). Thus, upon production of ECP2, PVX becomes avirulent on the two accessions of *N. paniculata*, indicating that ECP2-mediated recognition, possibly due to the presence of a *Cf-ECP2* homologue, arrests viral spread in these plants. Specific recognition of ECP2 by the nonhost plant *N. paniculata* implies either that a pathogen of this plant produces a factor sharing similarity to ECP2, or this ability to specifically recognize ECP2 has been generated more or less randomly. Isolation and sequencing of the *Cf-ECP2* gene from tomato and the gene mediating ECP2 recognition in tobacco should reveal to what extent the two genes are related.

The observation that specific recognition of a protein produced by PVX results in HR that is able to restrict systemic spread of the recombinant virus has also been described for the AVR9-*Cf-9* combination (54). Introduction of cosmids carrying *Cf-9* into tobacco and potato, followed by injection of AVR9, resulted in

a rapid HR, which indicated that in these plants the *Cf-9* gene functions normally and the Cfg-mediated signal transduction chain leading to HR is conserved (41). Inoculation of PVX::*Avr*9 onto *Cf-9*-transgenic tobacco resulted in resistance of the plant against the virus, whereas PVX derivatives producing AVR9 mutants with reduced elicitor activity were able to spread and caused systemic necrotic and/or mosaic symptoms (54).

Other Extracellular Proteins of *C. fulvum*

In addition to ECP2, the ECP1 protein was found to be a virulence factor of *C. fulvum* (65). A PVX::*Ecp*1 recombinant was constructed and used to screen a "core" collection of 40 wild accessions of *L. pimpinellifolium*. This wild currant tomato can easily be crossed to *L. esculentum* and has provided most of the resistance traits that are currently exploited in cultivated tomato. From each accession four individuals were inoculated with the recombinant virus, and plants were scored for the development of systemic necrotic symptoms seven to ten days after inoculation. Among the four inoculated plants of accession Lp#18, one plant was identified that showed systemic HR upon inoculation with PVX::*Ecp*1 (R Laugé, P Goodwin, PJGM De Wit & MHAJ Joosten, unpublished data). This plant was selfed and inoculation of the F1 with PVX::*Ecp*1 resulted in the development of systemic HR in all plants that were analyzed. However, inoculation of progeny with the ECP1-minus strain of *C. fulvum* showed that the plants were still resistant, which suggests the presence of additional *Cf* genes. Injection of AF isolated from a compatible interaction involving this ECP1-minus strain in leaflets of the progeny plants still resulted in HR, which indicates that these plants do indeed respond to additional avirulence factors of the fungus (R Laugé, P Goodwin, PJGM De Wit & MHAJ Joosten, unpublished data).

Besides ECP1 and ECP2, three additional extracellular proteins, ECP3, ECP4, and ECP5, that originate from *C. fulvum* have been purified from AF obtained from a compatible interaction (R Laugé, P Goodwin, PJGM De Wit & MHAJ Joosten, unpublished data). Although all three proteins are secreted in high amounts by *C. fulvum* upon colonization of intercellular spaces of tomato leaves, it is not yet known whether these ECPs are essential virulence factors, as targeted gene disruption and pathogenicity assays have yet to be performed. Except for ECP3, the encoding cDNAs for all ECPs have been cloned. All ECPs have a relatively low molecular weight (7 kDa to 12 kDa), contain an even number of cysteine residues (4 to 8), and, like the AVRs, do not show similarity to protein sequences present in databases (49, 105, 107). The cysteine residues could all be involved in disulfide bridges, which would ensure stability of the proteins upon secretion into the apoplast. In this regard, they are very similar to the AVR4 and AVR9 proteins, which suggests that they might cause avirulence of *C. fulvum* on particular tomato genotypes.

To determine whether tomato genotypes exist that are able to specifically recognize ECP3, ECP4, or ECP5, plants were either inoculated with PVX constructs

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harboring the cDNAs encoding the ECPs or injected with purified ECPs. Among a collection of tomato genotypes originating from various breeding programs and selected for resistance against *C. fulvum*, plants responding with HR to ECP3 and ECP5 were identified. Extension of the screen to a representative "core" collection of *L. pimpinellifolium* resulted in the identification of plants responding to each of the three ECPs (R Laugé, P Goodwin, PJGM de Wit & MHAJ Joosten, unpublished data). Selfing of the responding plants again resulted in an F1 of which the whole progeny tested showed HR upon exposure to the ECPs.

Thus, it appears that for all ECPs of *C. fulvum* isolated so far, *Lycopersicon* species exist that specifically respond with HR. All these stable cysteine-rich proteins secreted by the fungus can therefore be regarded as being "antigenic" for the *Lycopersicon* population.

RESISTANCE GENES IN TOMATO AGAINST *C. FULVUM*

Isolation and Characterization of the *Cf-2, Cf-4, Cf-5,* and *Cf-9* Genes

The *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* genes have been introgressed from wild tomato species into the tomato cultivar Money Maker (MM), resulting in the NILs MM-Cf2, MM-Cf4, MM-Cf5, and MM-Cf9, respectively. Mapping studies based on crosses between these NILs and *L. penellii* revealed that these *Cf* genes are present at two distinct loci in the tomato genome. *Cf-4* and *Cf-9* are closely linked on the short arm of chromosome 1, whereas *Cf-2* and *Cf-5* map at identical locations on the short arm of chromosome 6 (3, 46).

The four *Cf* genes were isolated following two different approaches. The *Cf-9* gene was isolated by transposon tagging, based on a screen that selected for loss of AVR9 recognition (48). The other three *Cf* genes were isolated by positional cloning that included complementation for resistance to particular races of *C. fulvum* by cosmids carrying the candidate *Cf* gene (29, 30, 101). All *Cf* genes are predicted to encode extracytoplasmic, membrane-anchored glycoproteins that contain many leucine-rich repeats (LRRs). These LRRs, of which each Cf protein contains a varying number, are thought to adopt a rod-like α-helical structure that could act as a site for binding of proteinaceous ligands or interaction with other LRR-containing proteins (57). The extracytoplasmic location of this binding site suggests that the Cf proteins can directly act as extracellular receptors for elicitors secreted by *C. fulvum*. In Cf4 and Cf9, however, the C terminus contains a KKxx sequence motif (100) that could be involved in retrieval of the processed Cf protein from the Golgi to the ER, suggesting a cytoplasmic localization (87). To study the exact cellular localization of Cf9, Benghezal & Jones (4a) fused the sequence encoding green fluorescent protein of jellyfish (GFP) to *Cf-9* and expressed the construct in yeast and tobacco protoplasts. By applying confocal light microscopy, they observed that most of the fusion protein was located at the ER. These

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experiments suggest that the Cf9 protein is targeted to the ER owing to the functionality of the KKxx motif, because a GFP-tagged Cf9 protein that contains a mutation in the KKxx motif (KSxx) was directed to the plasma membrane. Transfer of the fusion construct to tomato and *Arabidopsis thaliana* should reveal whether the KKxx motif is functional in these plants. Injection of AVR9 into leaflets of transgenic tomato containing GFP-Cf9 revealed that both N- or C-terminal fusions of GFP to Cf9 are functional as specific necrosis was observed. If the KKxx motif is functional and indeed results in total retrieval of the Cf9 protein to the ER, a direct extracellular interaction between Cf9 and AVR9 and also between Cf4 and AVR4 is not likely. This would imply that the interaction occurs in the cytoplasm, possibly as a result of endocytosis of the AVR protein. If only partial ER retrieval of the Cf9 protein occurs owing to masking of the KKxx motif by interacting proteins, the amount of Cf9 that is targeted to the plasma membrane could still be sufficient for perception of the AVR9 signal at the cell surface. However, inoculation of MM-Cf4 or MM-Cf9 plants with PVX::*Avr*4 or PVX::*Avr*9, respectively, only results in HR when the elicitor proteins contain a signal sequence for extracellular targeting, indicating that perception of the elicitors occurs extracellularly (R Laugé, G Honée, PJGM De Wit & MHAJ Joosten, unpublished data).

Sequence comparisons suggest that the variability of the Cf proteins resides in the N-terminal half (100). Although the C-terminal halves of the Cf4 and Cf9 proteins are nearly identical, their N-terminal halves show distinct differences at the amino acid level. The Cf2 and Cf5 proteins are also very similar in their C-terminal domains, with most variations in the N-terminal part. Typical sequence differences that distinguish the various Cf proteins correspond to the variable amino acid residues present in the highly conserved xxLxLxx ß-strand/ß-turn motif, where L represents a leucine or any aliphatic amino acid, and x can be any amino acid (38, 100). If there is a direct interaction between elicitors secreted by *C. fulvum* and Cf proteins, these variable amino acids might determine the specificity of ligand binding (38). This would explain why the various *Cf* genes have different resistance specificities against various strains of *C. fulvum*. Mutagenesis of various regions and domain exchange experiments between the different Cf proteins might reveal which areas in the proteins are determinants of recognition specificity (RAL Van der Hoorn, R Roth & PJGM De Wit, unpublished data; CM Thomas & JDG Jones, unpublished data). The functionality of the different constructs can be studied by performing an *Agrobacterium tumefaciens* transient transformation assay (ATTA). For this assay, *A. tumefaciens* containing the mutated *Cf* gene construct is either infiltrated into leaves of transgenic tobacco expressing *Avr*4 or *Avr*9 or is co-infiltrated with *A. tumefaciens* containing the *Avr* gene constructs. Alternatively, both the mutated *Cf* gene and its matching *Avr* gene can be present in one construct.

The C-terminal domains of the four Cf proteins are highly conserved. As the cytoplasmic part of all Cf proteins is very small (a predicted 21 to 37 amino acids), it is unlikely to function directly in signal transduction. Therefore, it is envisaged that the conserved (extracellular) C-terminal half interacts with an

additional transmembrane protein. This interacting protein should have the necessary cytoplasmic domain to transduce a signal to the interior of the cell (see below).

The *Cf* Gene Organization Allows Sequence Diversification and Generation of New Recognitional Specificities

Southern blot analysis and mapping studies have shown that all *Cf* genes are organized as clustered gene families (100, and references therein). The functional *Cf* genes are surrounded by gene homologues that have been designated *Hcr*, for homologues of *Cladosporium fulvum* resistance genes.

MM-Cf2 plants contain two functional *Cf-2* resistance genes (*Cf-2.1* and *Cf-2.2*) that differ in only three amino acids near the C terminus. There is a third homologue present (*Hcr2-2A*), which does not appear to be functional in providing resistance against *C. fulvum* (30). MM-Cf5 plants also contain members of the *Cf-2* multigene family (*Hcr2-5*) (29). Besides the functional *Cf-5* gene itself (*Hcr2-5C*), three additional gene homologues are present, *Hcr2-5A*, *B*, and *D*, that contain between 25 and 38 LRRs.

On the introgressed region of the short arm of chromosome 1, several genes are present that are homologous to the *Cf-9* resistance gene (*Hcr9*s). In this region from MM-Cf9 plants, five homologues are present: *Hcr9-9A* to *Hcr9-9E*. Within this cluster, *Hcr9-9C* is the functional *Cf-9* gene, as this homologue enables plants to recognize the AVR9 elicitor (80). In MM-Cf4 plants the situation is similar; there are five homologues present, *Hcr9-4A* to *Hcr9-4E*, of which *Hcr9-4D* is the functional *Cf-4* gene that allows AVR4-induced HR to occur (101).

The identification of chimeric gene clusters in *Cf-2/Cf-5* and *Cf-4/Cf-9* test cross plants indicated that the clustered organization of the *Cf* genes allows chromosomal mispairing and unequal crossing-over to occur. At the *Cf-2/Cf-5* locus, an intragenic recombination event was identified that resulted in a chimeric gene, consisting of the sequence encoding the signal peptide of the *Cf-2.2* gene fused to the sequence encoding the mature part of the *Hcr2-5B* protein (29). At the *Cf-4/Cf-9* locus, several intergenic recombination events have been identified (101); more detailed analysis of the *Hcr9* family members revealed that extensive sequence exchange has occurred between the individual *Hcr9*s (80).

One of the first indications that sequence diversification might generate new resistance specificities was obtained by Takken and coworkers (97). They performed a transposon-tagging experiment in which the *Activator* (*Ac*)/*Dissociation* (*Ds*) system was used to isolate the gene that confers resistance to race 5 of *C. fulvum*, a strain that produces the AVR4 elicitor. They observed that tagged plants that had become susceptible to this race contained a *Ds*-element in the *Cf-4* locus. These plants, however, still showed systemic HR upon inoculation with PVX::*Avr*4. Although the HR was delayed when compared to the response of MM-Cf4 wild-type plants, this result indicated that the *Cf-4* resistance gene itself (*Hcr9-4D*) was still intact. Detailed analysis of the *Ds*-tagged plants indicated that the transposon had inserted into the *Hcr9-4E* homologue, which showed

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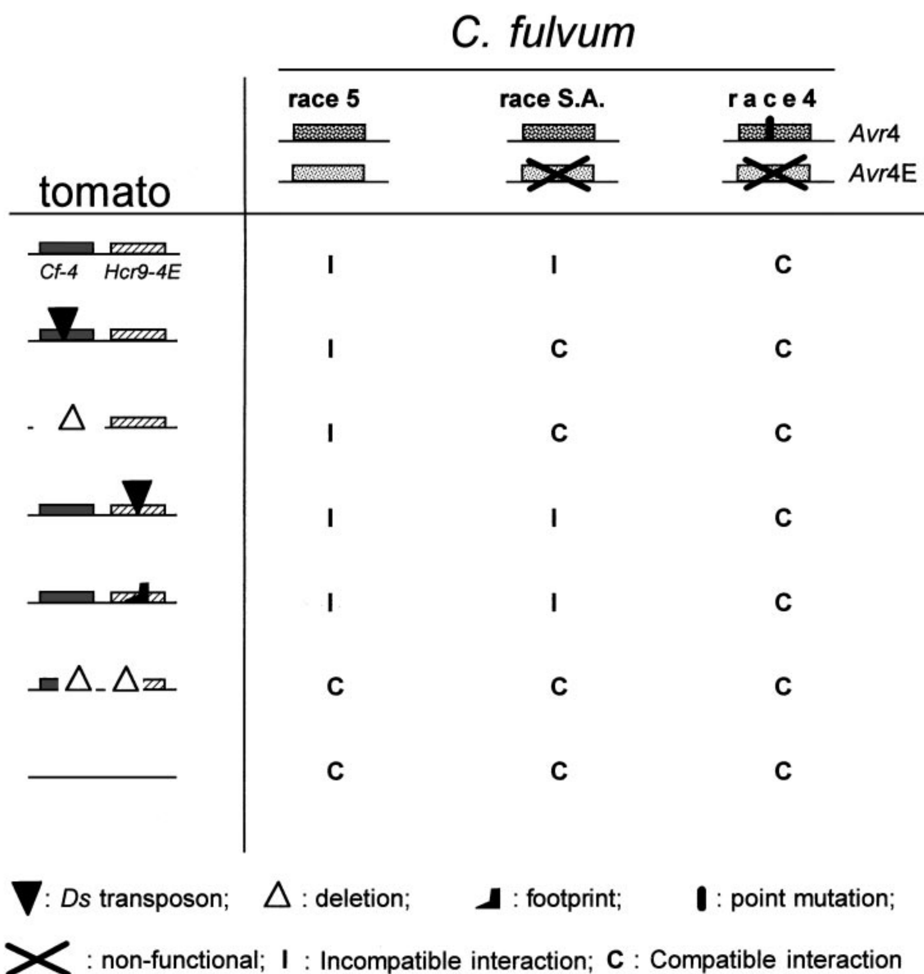

Figure 3 Outcome of the interaction between three different races of *C. fulvum* and mutants of MM-Cf4 tomato generated by transposon mutagenesis. The tomato mutants either carry a transposon (*Ds*) insertion or a footprint, or contain a deletion of one or two of the functional *Hcr9-4* homologues (*Hcr9-4D*, which is the *Cf-4* gene itself, or *Hcr9-4E*) present at the *Cf-4* locus. Race S.A.: strain of *C. fulvum* originating from South America. See text for details.

that this gene also confers resistance against *C. fulvum* (97) (Figure 3). The *Ds* transposon insertion into the *Hcr9-4E* homologue resulted in a nonfunctional gene and delayed the AVR4-induced HR. This appeared to be sufficient to render the plant susceptible to the fungus. The finding that insertion of a transposon in one homologue also affects the functionality of another gene homologue could be the result of cross-talk between the two genes, possibly via a mechanism of sense- or antisense-suppression (92). Indeed, the presence of promoter-like elements in

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the inserted *Ds* element might lead to sense or antisense transcription of flanking sequences (88).

The observation that two homologues present at the *Cf-4* locus are functional in generating resistance against race 5 of *C. fulvum* was further supported by the analysis of plants containing various deletions or insertions at this locus (Figure 3). Progeny of a tagging experiment in which plants containing the *Ds* element inserted between *Cf-4* and *Hcr9-4E* were crossed to transgenic MM-Cf0 plants that constitutively produced the AVR4 elicitor (FLW Takken, D Schipper, J Hille & HJJ Nijkamp, unpublished data). In all cases, the plants that showed normal growth contained a deletion or transposon insertion that had affected the *Cf-4* gene itself. Since in most of these plants the *Hcr9-4E* gene was still intact, this gene is clearly not involved in AVR4 perception. Inoculation of progeny plants with race 5 of the fungus showed that the presence of a functional copy of either the *Cf-4* gene itself or the *Hcr9-4E* homologue was sufficient to confer resistance. This indicated that apart from AVR4, race 5 produces an additional avirulence factor (AVR4E) that is perceived by *Hcr9-4E*. Plants that lack both the *Hcr9-4D* and *-4E* homologues were fully susceptible to race 5 of *C. fulvum*, which indicates that the *Hcr9-4A*, *-4B* and *-4C* homologues are not involved in resistance of tomato against this race.

Inoculation of transgenic MM-Cf0 plants that express the individual *Hcr9-4* homologues with race 5 of *C. fulvum* confirmed observations made with the transposon-tagged and deletion mutants. Plants expressing the *Hcr9-4A*, *-4B*, and *-4C* homologues were as susceptible as MM-Cf0 plants, while the *Hcr9-4D*- and *-4E*-expressing plants were fully resistant to race 5 (see also Figure 3) (FLW Takken, CM Thomas, MHAJ Joosten, C Golstein, N Westerink, J Hille, HJJ Nijkamp, PJGM De Wit & JDG Jones, unpublished data). Inoculation of the individual transgenic plants with various strains showed that those strains virulent on MM-Cf4 plants, containing the complete *Cf-4* cluster, are also virulent on all five plants expressing the individual *Hcr9-4* homologues (see also Figure 3). One strain, which originates from South America (race S.A.) and which is avirulent on MM-Cf4 plants, colonized plants expressing *Hcr9-4E*, whereas it was avirulent on plants expressing *Hcr9-4D* (see also Figure 3). This suggests that this strain still produces AVR4 but has circumvented *Hcr9-4E*-mediated resistance, owing to the lack of a functional AVR4E elicitor protein. Loss of the functional copy of *Avr*4 should result in virulence of this strain on plants containing the complete *Cf-4* cluster. This is currently being tested by performing a targeted disruption of the *Avr*4 gene in this strain and as a control also in an isolate of race 5. Loss of the *Avr*4 gene should result in a transgenic race 5 that is still avirulent on MM-Cf4 plants, while race S.A. should become virulent on such plants (N Westerink, PJGM De Wit & MHAJ Joosten, unpublished data).

Injection of AF isolated from a compatible interaction involving race 5 induces HR in plants that contain only the *Hcr9-4E* homologue. A 10-kDa fungal protein, which induces HR and therefore might represent the putative avirulence factor AVR4E, was purified and partially sequenced. Currently, the *Avr*4E gene is being isolated (N Westerink, PJGM De Wit & MHAJ Joosten, unpublished data). It will

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be interesting to know whether the *Avr4E* gene is absent or mutated in strains of *C. fulvum* that have circumvented *Hcr9-4E*–mediated resistance.

At the *Cf-9* locus, sequence exchanges between the various clustered homologues have also resulted in the generation of additional resistance specificities. Inoculation of MM-Cf9 plants with two mutant strains obtained by disruption of *Avr9* in races 4 and 5 of *C. fulvum*, resulting in races 4Δ9 and 5Δ9, respectively (72), revealed that the plants still show weak resistance (63). This indicates that on the *Cf-9* introgression segment one or more genes are present that confer additional resistance specificities. Transformation of the individual *Hcr9-9A*, *-9B*, *-9C*, *-9D*, or *-9E* genes, which reside on the introgressed *Cf-9* segment, into MM-Cf0 plants proved that, in addition to the *Cf-9* gene itself, *Hcr9-9B* also provides resistance to races 4 and 5 of *C. fulvum* (80). This resistance is independent of the production of AVR9 by the fungus, as race 5Δ9 was also unable to sporulate on the transformant expressing *Hcr9-9B*. Plants containing the *Hcr9-9E* homologue showed partial resistance resulting in delayed sporulation upon inoculation with races 4, 5, or 5Δ9. Thus, *Hcrg-gE* might encode a protein that allows the plant to recognize an additional avirulence determinant of the fungus.

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Analogies between *Cf* Gene Diversification and the Versatility of the Innate Immune System Found in Mammals

Numerous *R* genes have now been cloned from various plant species. In most cases, *R* genes are organized in clusters of homologues that allow sequence duplication, gene recombination, gene conversion, and diversifying selection to occur (38, 86). This observation is reminiscent of the situation of the major histocompatibility complex (MHC) of mammals (15, 16). The MHC is involved in cellular and humoral immune responses and spans a region of about 4 Mb of DNA (102). There are about 80 MHC genes, among which are several clustered genes with related functions in antigen processing and presentation (MHC class I and class II genes). Each individual carries only one specific set of MHC genes, which is often unique. At the population level, numerous different recognitional specificities are created, which is the main cause of rejection of transplanted organs, because the differences in MHC genes between donor and acceptor can be large. It appears that various MHC genes have duplicated, resulting in one copy that retains its original function and the additional copy diverging to gain a novel function. In addition, similar functions can be encoded by different MHC genes in diverse species (102). Analogous to this system, different *Hcr* genes in diverse plant species might encode similar recognition specificities. This might be the case for *Cf-ECP2* and the corresponding gene in *N. paniculata*. Among the allelic variants within the class I and class II molecules of the MHC, the most significant differences are based on amino acid substitutions of key residues in the peptide-binding groove. Similar to the variability in the N-terminal halves of the Cf proteins, non-synonymous mutations are more abundant than synonymous ones, which suggests the presence of positive selection on diversity (80, 102). Furthermore, shuffling of

existing amino acid motifs into new combinations, gene conversion, and recombination have also created new MHC loci. Mapping of MHC genes revealed that in the human genome several regions are present that contain related genes (55). To date, four so-called paralogous regions have been identified, containing clusters of genes with related copies in the MHC. It is suggested that the various regions arose as a result of chromosomal duplications (55). This situation is similar to the organization of the various *Cf* loci in the tomato genome; probably, these loci can also be regarded as paralogues that have evolved from chromosomal duplications (80, 100).

That plants contain a versatile recognition system for "non-self" proteins is supported by the observation that additional recognitional specificities are present at the *Cf-4* and *Cf-9* loci, as well as by the fact that plants responding with HR have been identified for all ECPs that have been isolated to date (R Laugé, P Goodwin, PJGM De Wit & MHAJ Joosten, unpublished data). The multiple recognition specificities conferred by sets of *Cf* genes are randomly scattered among the individuals within a population, ensuring survival of some individuals of the species upon attack by *C. fulvum*.

A continuous generation of new recognitional specificities within a population might give rise to individuals containing *Cf* alleles that allow recognition of "self-proteins" to occur. These individuals will normally be eliminated from the population, as systemic HR will be the result. If, however, this self-recognition only occurs with a low affinity or is induced by specific environmental conditions at certain developmental stages, it could also cause a phenotype that has been described as "disease lesion mimics." This phenotype has been found in such species as *A. thaliana*, maize, tomato, and barley (17, 45). In tomato, such a necrogenic phenotype is correlated with the presence of the *Cf-2* locus (62). However, Dixon and coworkers (30) observed that this phenotype is not dependent on the presence of *Cf-2* alone, as primary transformants of MM-Cf0 plants containing cosmids carrying the *Cf-2* gene did not show any necrosis. An additional dominant gene closely linked to the *Cf-2* locus is probably responsible for this phenotype. It is tempting to speculate that this linked gene is a *Hcr2-2* homologue that has evolved from the *Hcr2* cluster and allows recognition of a "self-protein" to occur.

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RECOGNITION OF AVIRULENCE FACTORS OF *C. FULVUM* BY TOMATO

Perception of Avirulence Factors

Binding studies have shown that both resistant and susceptible tomato genotypes contain a high-affinity binding site (HABS) for the AVR9 peptide in plasma membranes of mesophyll and suspension cells, suggesting that the Cf proteins are not the primary receptors of the elicitors (58). As such, the HABS could represent the target of AVR9 in its function as a putative virulence factor (Figure 4*a*). Binding

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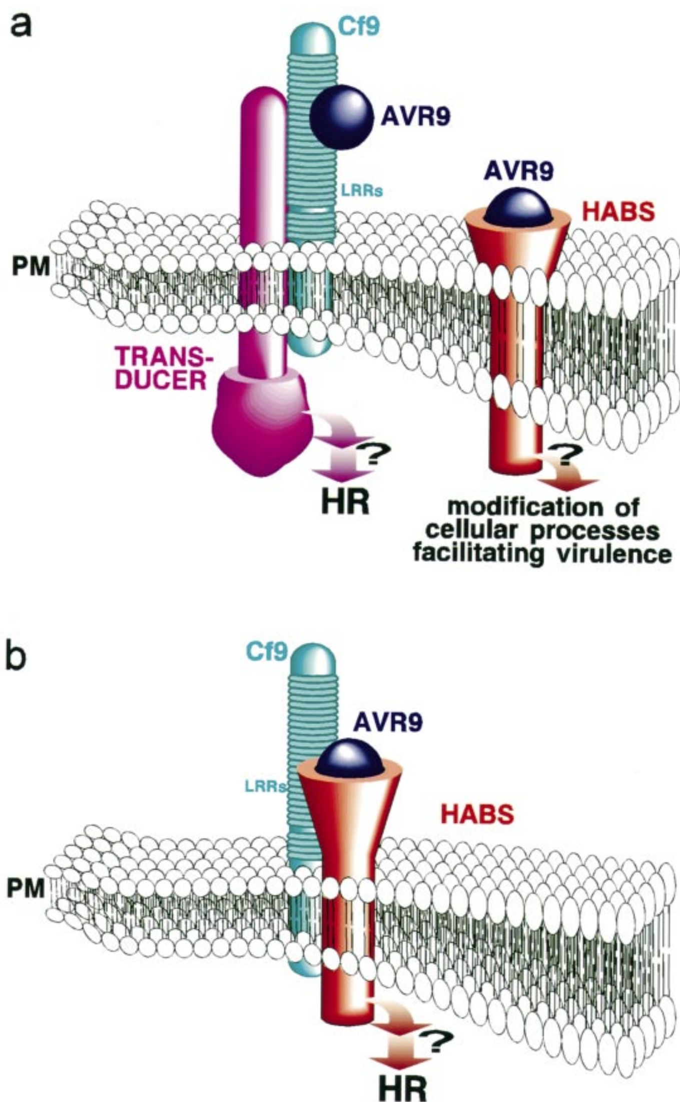

Figure 4 Three models of perception and signaling in the Cf9-AVR9-mediated HR (*a*, *b* and *c*) and a model of the mechanism by which CLAVATA (CLV) proteins regulate developmental processes in the shoot apical meristern of Arabidopsis (*d*). See text for details.

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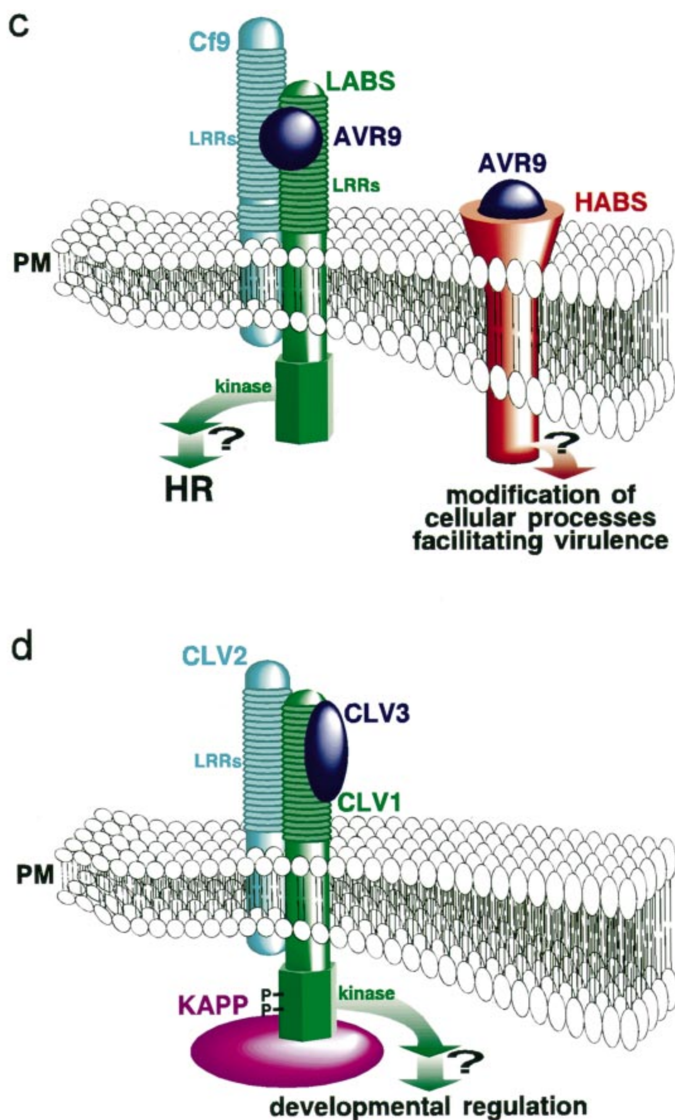

Figure 4 (*continued*) PM: plasma membrane; HABS: high-affinity binding site; LABS: low-affinity binding site; CLV1, CLV2, and CLV3: CLAVATA proteins.

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to the HABS alone does not provoke HR. The Cf9 protein, which is present only in MM-Cf9 plants, might be a low affinity binding site (LABS) for AVR9. This LABS, which cannot be detected in isolated plasma membranes also containing the HABS, could activate signal transduction, leading to HR via a transducer molecule that associates with the Cf9-AVR9 complex (60).

Alternatively, Cf proteins could represent factors that specifically interact with an elicitor-HABS complex (60) (Figure 4*b*). The AVR9-HABS complex, which does not have to be specific for MM-Cf9 plants, is expected to contain a domain that activates cytoplasmic signaling. In MM-Cf9 plants, the variable N-terminal half of the Cf9 protein could allow specific association with an AVR9-HABS complex. Binding of AVR9 to the HABS might activate the formation of a HABS-Cf9 protein heterodimer, which could subsequently result in conformational changes leading to further signal transduction into the cytoplasm. In MM-Cf0 plants in which the Cf9 protein is not present, a heterodimer cannot form and, consequently, signal transduction leading to HR does not occur. Ligand-induced receptor dimerization is essential to regulate the catalytic activity of receptor protein tyrosine kinases (PTKs) that interact with growth factors and subsequently transduce a signal (110). Signal transduction involves phosphorylation cascades that can eventually activate effector proteins such as enzymes or transcription factors. In addition, members of many other families of cell surface receptors undergo oligomerization upon specific binding of their ligand (110, and references therein). In tomato, the receptor itself appears to be specific for AVR9, because AVR4, for example, does not compete for binding to the HABS (58). Furthermore, a clear positive correlation has been found between HR-inducing activity and affinity of mutants of AVR9 to the HABS (60).

A third model is presented in Figure 4*c*. In this model, the HABS represents the "virulence" target of AVR9, whereas one or more AVR9 molecules can also bind to a signal-transducing receptor complex with low affinity. In this case, formation of a heterodimer between a transducing LABS and the Cfg protein might be essential for elicitor binding and subsequent generation of a cytoplasmic signal. In order to transduce a signal, the LABS should contain a cytoplasmic signaling component, since Cf9 itself lacks such a component. This can either be a cytoplasmic kinase that associates with the heterodimer or the kinase domain can be part of the C-terminal domain of the LABS that interacts with the Cfg protein upon elicitor binding. To allow interaction with the Cfg protein to occur, the LABS could also contain an LRR-rich domain in the extracytoplasmic region. A typical example of such an LRR, receptor-like kinase (LRR-RLK) is the protein encoded by the *Xa21* gene, which confers resistance of rice against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (90). PCR analysis, employing degenerate primers based on conserved motifs in the LRR and kinase domain of this putative receptor, revealed that several *Xa21*-like genes are present in tomato (96a), which suggests that heterodimerization of Cf proteins with a Xa21-like protein is feasible.

An increasing number of LRR-RLKs, of which many are involved in plant development, have been described in several plant species (4, 47). An example

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of such a receptor kinase is the CLAVATA1 (CLV1) protein of *A. thaliana*. It takes part in a complex regulatory mechanism that controls the balance between cell proliferation and cell differentiation in the apical shoot meristem (12, 66). Based on the expression pattern of *CLV1* within the shoot apical meristem, the CLV1 protein is thought to perceive positional information from surrounding cells that is then used to regulate meristem size. The extracellular domain of the CLV1 protein contains 21 LRRs, which might be involved in binding of a soluble apoplastic ligand (13) (Figure 4*d*). A likely candidate for the ligand of CLV1 is CLV3 (11). The *CLV3* gene encodes a protein of 96 amino acids; it contains a signal sequence for extracellular targeting of 18 amino acids and does not show significant homology to any protein sequence present in the databases. In contrast to the ECPs and AVRs secreted by *C. fulvum*, the mature CLV3 protein does not contain any cysteine residue (33). A third gene has been cloned that also plays a role in the CLV-regulated meristem development. This gene, *CLV2*, encodes a putative transmembrane protein (CLV2) that consists of 22 extracellular LRRs and a very short cytoplasmic domain (44a). Based on CLV1-, CLV2-, and CLV3 mutant phenotypes, CLV2 is thought to interact with the CLV1 protein upon binding of the CLV3 protein (55a) (Figure 4*d*). A fourth component of the pathway is a kinase-associated protein phosphatase (KAPP). This protein was found to directly interact with autophosphorylated CLV1 and appears to be a negative regulator of CLV1 action (94, 116). Recent studies based on the immunological analysis of cauliflower extracts revealed that the CLV1 protein is present in a signaling complex that requires CLV3 for its assembly (101a). In the complex, CLV1 is most likely present as a disulfide-linked heterodimer that contains either another LRR-RLK or the CLV2 protein, as was suggested before (55a). In addition to KAPP, a Rho GTPase-related protein was found to be present in the signaling complex (101a). This GTPase could be associated with the kinase domain of CLV1 and facilitate the triggering of a mitogen-activated protein kinase (MAPK) pathway. Such a mechanism to relay extracellular signals to intracellular downstream targets has been described for many receptor kinases in animal systems (69a).

The similarity between Cf9 and CLV2 suggests that the Cf proteins interact with an LRR-RLK protein to transduce an exogenously perceived signal, such as AVR9, to the cytoplasm. Thus, the situation for Cf proteins could be analogous to the mechanism by which the CLV proteins mediate transduction of an endogenous signal (compare Figures 4*c* and 4*d*). Similar to endogenous CLV3, exogenous AVR9 could interact directly with a CLV1 analogue, which has to interact with Cf9, a CLV2 analogue, to eventually transduce a signal. Further extrapolation of the analogy suggests that a Rho GTPase-related protein could be involved in channeling the signal into a MAPK pathway eventually leading to HR. Interestingly, Romeis and colleagues (84a) and CF de Jong, H van Leeuwen, G Honée, MHAJ Joosten & PJGM de Wit (unpublished data) have shown that the AVR9-Cf9-mediated response involves the activation of two MAP kinases (see below). Also in this case, downstream signaling from the kinase domain of a CLV1 analogue to the GTPase could be regulated by a KAPP. Thus, a comparison of the CLV-

mediated developmental regulation and AVR9-Cf9-mediated HR reveals striking analogies. During evolution, gene families involved in regulation of development, of which the products respond to endogenous signals, were perhaps also recruited to perceive and transduce extracellular signals produced by attacking pathogens. Since perception of such an extracellular signal leads to HR, the transduced signal must eventually be funneled into a separate pathway. As mentioned earlier, the secreted proteins of *C. fulvum* all contain an even number of at least four cysteine residues (49, 105, 107), whereas the mature CLV3 protein does not contain any cysteine residue (33). It is possible that disulfide bonds between the cysteine residues present in the ECPs and AVRs of *C. fulvum* provide sufficient rigidity and stability, allowing the proteins to pass the apoplast to reach the complementary receptor (complex) present on the plasma membrane of the host cells. Deletion of crucial disulfide bonds loosens the structure of the secreted proteins and makes them prone to proteolytic degradation, a phenomenon that appears to form the basis for circumvention of *Cf-4*-mediated resistance (53). As CLV3 is secreted by the plant cells themselves, the route to the receptor complex is much shorter and therefore high stability of the protein is less crucial (53). The putative instability of the CLV3 protein might allow subtle triggering of downstream signaling processes in only a limited number of cell layers, as the ligand will be degraded soon after secretion and the ligand-receptor complex will be present for only a short time. Signals provoked by the stable proteins secreted by *C. fulvum* might be more persistent, which could also explain why these are eventually funneled into a pathway leading to HR.

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Defense Responses Induced after Elicitor Perception

May and coworkers (76) isolated AF from a compatible interaction involving race 5 of *C. fulvum* and made a preparation containing the AVR2 and AVR9 elicitors that are secreted by this race. Injection of the elicitors into cotyledons of MM-Cf2 or MM-Cf9 seedlings, which are resistant against race 5 of *C. fulvum*, resulted in a strong oxidative burst, whereas susceptible MM-Cf0 seedlings hardly responded. This oxidative burst, which involves the production of active oxygen species (AOS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), is regarded as one of the first responses to be generated upon recognition of an attacking pathogen (61). AOS, which could exert direct fungitoxic activity, induce strengthening of walls of the surrounding plant cells and contribute to the activation of defense-related genes. Upon injection of fractions containing elicitors into resistant cotyledons, the formation of O_2^- was evident within two to four hours (76). As a consequence, lipid peroxidation leading to cell membrane damage was observed, a phenomenon also described by Peever & Higgins (81). This membrane damage probably marks the onset of HR and was reflected by increases in solute leakage from discs that were cut from the injected tissue. Furthermore, transient accumulation of free salicylic acid and a burst in the production of ethylene was observed within 24 h (40). Both salicylic acid and ethylene are thought to enhance the generation of

defense responses at the site of pathogen recognition, futhermore, these substances are also involved in mediating systemic defense responses (32, 120).

Recently, it has been shown that the AOS act in concert with nitric oxide (NO) in inducing HR. In an incompatible interaction between tobacco and TMV, NO synthase activity had increased (31), whereas inhibition of NO synthesis reduced HR normally induced in *A. thaliana* upon inoculation with an avirulent strain of the bacterium *Pseudomonas syringae* pv. *maculicola* (19). The occurrence of DNA fragmentation upon HR suggests that programmed cell death occurs upon recognition of an avirulent pathogen (2).

Cell suspensions of MM-Cf9 tomato do not respond to the AVR9 elicitor, whereas aspecific elicitors, such as the Pmg elicitor of *P. sojae* (formerly called *P. megasperma* f.sp. *glycinea*), induced all characteristic defense-related responses (43). In addition to the induction of an oxidative burst and the expression of defense-related genes such as 1,3-ß-glucanases and chitinases, strong alkalization of the medium occurred upon addition of the Pmg elicitor. The alkalization reflects the occurrence of ion fluxes, involving uptake of H^+ and Ca^{2+} by the cells (as was also confirmed by the demonstration of an increase in the amount of cell-associated $^{45}Ca^{2+}$) and efflux of K^+ and Cl^-. These ion fluxes are thought to represent an early signaling event that eventually activates signal transduction cascades (8, 32, 120). Although the pathway involved in elicitor-induced defense responses is functional, undifferentiated suspension cells and also callus tissue of Cfg tomato probably do not contain a functional *Cf-9*–mediated signal transduction pathway. The ability of AVR9 to specifically induce defense responses in MM-Cf9 tomato suspension cells is thought to be suppressed or absent, in contrast to MM-Cf9 mesophyll cells that respond quickly (43).

Surprisingly, cell suspensions generated from transgenic, *Cf-9*-expressing tobacco plants showed specific responses upon treatment with the AVR9 elicitor (82; CF de Jong, JB van Leeuwen, MHAJ Joosten, G Honée & PJGM de Wit, unpublished data). The AVR9 peptide induces an oxidative burst at very low concentrations ($EC_{50} = 2.5$ nM), whereas alkalization of the medium required much higher concentrations of AVR9 ($EC_{50} = 130$ nM). The differences in necrosis-inducing activity of the various mutants of AVR9, which had been tested in the PVX-expression system and had been chemically synthesized (59, 60, 71), correlated with their capacity to induce an oxidative burst and alkalization in cell suspensions. This suggests that the severity of necrosis induced in leaflets of MM-Cf9 plants upon injection with wild-type AVR9 and the various mutants correlates with the levels of oxidative burst and ion fluxes induced in transgenic *Cf-9* tobacco cell suspensions. Piedras and coworkers (82) also showed that Cf9- and AVR9-dependent responses occurred in *Cf-9*–containing tobacco cell suspensions and that H_2O_2 is the most abundant AOS present. As DPI, a specific inhibitor of flavin-containing enzymes, was able to completely block AOS accumulation at low concentrations, it was concluded that an NADPH oxidase complex is responsible for the generation of the oxidative burst. The involvement of NADPH oxidase in elicitor-induced defense responses has also been suggested by Xing and colleagues (118), who showed that assembly of an active oxidase complex at the plasma

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membrane occurred upon treatment of MM-Cf4 or MM-Cf5 tomato cell suspensions with AF preparations containing specific elicitors of *C. fulvum*. The NADPH oxidase activity of plasma membrane preparations significantly increased when a cell suspension was treated with the corresponding elicitor. However, they did not perform measurements on intact cells, nor did they show whether AOS were actually produced. The activated NADPH oxidase complex transfers electrons from NADPH to oxygen, resulting in the formation of superoxide, which is readily converted into H_2O_2. Increased oxygen uptake in an elicited, *Cf-9*-containing tobacco cell suspension, possibly due to the generation of oxygen radicals, was indeed shown by Piedras and coworkers (82). Furthermore, they showed that the uptake of Ca^{2+} is essential to generate AOS, indicating that signal transduction leading to the oxidative burst is Ca^{2+}-dependent.

The importance of an increased concentration of cytosolic free Ca^{2+} to induce HR in cowpea upon attack by an avirulent strain of the cowpea rust fungus, *Uromyces vignae*, has been shown in intact tissue by Xu & Heath (119). They found a transient accumulation of free Ca^{2+} in epidermis cells of resistant plants, which was initiated at the time the fungus grew through the cell wall. Ca^{2+} levels were back to normal by the time the fungus grew within the cell lumen. By this time, the process of hypersensitive death of the invaded cell was initiated, which restricted fungal growth and resulted resistance. Ca^{2+} channel inhibitors prevented an increase of free cytosolic Ca^{2+} to occur and also significantly delayed the onset of HR. In susceptible plants there were no changes in free Ca^{2+} levels upon penetration and subsequent intracellular growth of the fungus, and HR was not observed.

In addition to the induction of ion fluxes, an oxidative burst, and alkalization of the medium, elicitation of *Cf-9*–containing tobacco cell suspensions with the AVR9 peptide also causes a rapid and transient activation of two MAP kinases of circa 48 kDa in molecular weight. They are thought to be located downstream of a Ca^{2+} channel and might be involved in a phosphorylation cascade (84a). The activation of these kinases might not be specific for the AVR9-Cfg–mediated response, as Suzuki & Shinshi (96) reported that treatment of tobacco cells with an undefined elicitor originating from the cell walls of *P. infestans* activates a 47-kDa kinase. A similar observation was made by Lebrun-Garcia et al (69), who found activation of two MAP kinases (50 and 46 kDa) upon elicitation of a tobacco cell suspension with the elicitin cryptogein of *P. cryptogea*. The activation was Ca^{2+}-dependent, required protein phosphorylation, and was independent of the production of AOS. The target molecules of the MAP kinase pathway are not yet known.

EXPLOITATION OF *Cf* GENES AND MATCHING *Avr* GENES IN MOLECULAR RESISTANCE BREEDING

Over a period of five years, several plant resistance genes have been cloned that provide race-specific resistance not only against viral, bacterial, or fungal pathogens but also against nematodes and even insects. The elucidation of the basic structure of the *R* gene-encoded proteins and, in some cases, the corresponding avirulence

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factors has provided clues about the mechanism by which such gene pairs can confer resistance against a pathogen. Detailed fundamental knowledge on the perception of avirulent pathogens, signal transduction, and subsequent activation of host defense responses that eventually result in restriction of pathogen spread and resistance is growing rapidly. However, the exploitation of *R* genes in breeding for resistance against pathogens does not require detailed information on all *R* gene–dependent resistance processes. For example, introduction of an *R* gene conferring resistance against particular races of a pathogen into a susceptible genotype renders these plants resistant to those races of the pathogen (30, 74). Thus, it can be concluded that in susceptible plants the *R* gene is the only lacking component to confer resistance. Inter species transfer of an *R* gene to another natural host of a pathogen allows a broader application of such an *R* gene in resistance breeding (41, 85, 99, 115). For instance, transfer of the *Pto* gene from tomato, conferring resistance to *AvrPto*-containing strains of the bacterium *Pseudomonas syringae* pv. *tomato*, to tobacco enhances its resistance to this bacterium (85, 99). Transfer of the *Cf-9* gene to tobacco and potato allows these plants to respond specifically to the AVR9 elicitor, again showing that the complete signal transduction pathway is retained in these plants (41). *C. fulvum*, however, is not a pathogen of these plants, which limits the use of such a specific *R* gene. As described above, the *Cf-ECP2* gene of tomato confers resistance against *C. fulvum* through recognition of the conserved virulence factor ECP2 that is produced by all strains of the fungus (64). Some accessions of *N. paniculata*, which is not a host for this fungus, also appeared to specifically recognize the virulence factor. If an ECP2-like protein, or a virulence factor containing a similar epitope, were of general importance for (fungal) pathogens, the *Cf-ECP2* gene might provide durable resistance against a variety of pathogens.

The availability of *Cf-4* and *Cf-9* and their corresponding *Avr* genes *Avr*4 and *Avr*9, respectively, allows expression of both the *Cf* gene and the matching *Avr* gene in the same plant. Combining *Cf-9* and *Avr*9 in one plant by crossing MM-Cf9 plants with MM-Cf0 plants that constitutively express *Avr*9, due to the presence of a *35S:Avr*9 transgene, results in death of the offspring at the seedling stage (37) (Figure 5*a*). This phenomenon provided an efficient assay to isolate the *Cf-9* gene by maize *Ac/Ds* transposon tagging, as offspring that contained a transposon-inactivated *Cf-9* gene developed normally (48). This approach allowed to obtain plants that contain *Ac*, *35S:Avr*9, as well as *Ds*-inactivated *Cf-9*. These plants might exhibit enhanced resistance to pathogens, as somatic excision of the *Ds*-element reactivates the *Cf-9* gene and allows recognition of the AVR9 peptide, resulting in HR (Figure 5*b*). If this excision occurs in a coordinated manner at several sites throughout the plant, plant defense responses might be systemically activated (41). This so-called genetically engineered acquired resistance (GEAR) could provide a method to protect plants against pathogens similar to the natural process whereby avirulent pathogens induce the development of systemic "micro-HRs" (2). Another approach, suggested by Hammond-Kosack and coworkers (41), consists of expressing weak alleles of both genes, for example, a mutated form of *Cf-9* (39) and *Avr*9 (59) that induce only very weak responses upon combination

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in one plant. This should result in a constitutive, systemic expression of a weak defense response in the plant. One of the main drawbacks of both approaches is that uncontrolled expression of the transgenes, even without the presence of a pathogen, can easily cause detrimental effects on the plants. HR will appear throughout the plant, resulting in the formation of spreading necrotic lesions and severe growth retardation or even death of the plant. Such uncontrolled suicide phenomena must be overcome before this approach can be followed in molecular resistance breeding of important crop plants.

An alternative approach that might circumvent all these problems is the two-component sensor system that was originally proposed by De Wit (21, 22). This system is also based on simultaneously expressing the *Cf* gene and matching *Avr* gene. However, in this case, the *Avr* transgene is expressed only at the time and site of infection by a pathogen. This well-defined expression is the result of placing the ORF of the *Avr* gene under transcriptional control of a pathogen-inducible promoter. Infection by a pathogen activates the promoter and triggers a local HR, which confines the invading pathogen. A good candidate for such a promoter is the promoter of the defense gene *gst1* of potato (75). This gene encodes a glutathione *S*-transferase, and a 273-base-pair fragment of its promoter is sufficient to mediate a localized and fast transcriptional activation of the hybrid gene in response to infection by *P. infestans*. The promoter is not activated by abiotic stimuli, such as wounding, heat shock, or changes in light intensity. Expression analysis of a fusion of this fragment to the GUS reporter gene in potato showed that the fragment also allows localized transcriptional activation in response to infection by various other pathogens, while background levels in healthy tissues remained low (95). Transformation of MM-Cf9 plants with the *Avr*9 gene placed under transcriptional control of this promoter fragment (Figure 5*c*) yielded plants that showed increased resistance to *C. fulvum* and *Oidium lycopersicum* (M Stuiver & G Honée, unpublished data). Small necrotic lesions, which mimicked the natural situation in which the recognized elicitor is produced by the avirulent pathogen, were observed at the infection sites.

These preliminary results show that this approach is feasible to obtain resistance against a broad range of pathogens. Apart from the *Cf-9-Avr*9 and *Cf-4-Avr*4 gene pairs other *R-Avr* gene pairs, when cloned, can be introduced in a construct that allows constitutive expression of the *R* gene and inducible expression of the *Avr* gene. This "cassette" can then be introduced into several plant species, rendering them resistant against a broad range of pathogens (Figure 5*d*). However, the prerequisite that the invading pathogen should be arrested as a result of HR probably limits this approach to obligate and biotrophic pathogens. To be able to use the "cassette" in various plant species, the signal transduction cascade leading to localized HR should be present and should be activated by the *R* gene product upon encountering the matching elicitor in these plants. In addition, further optimization of the tight regulation of the promoter will be needed, as activation of the *gst1* promoter fragment is not fully restricted to sites of infection (95). Further restriction and localization of HR could be obtained by placing *Avr*9 and *Cf-9* under control of two different pathogen-inducible promoters, which could tighten

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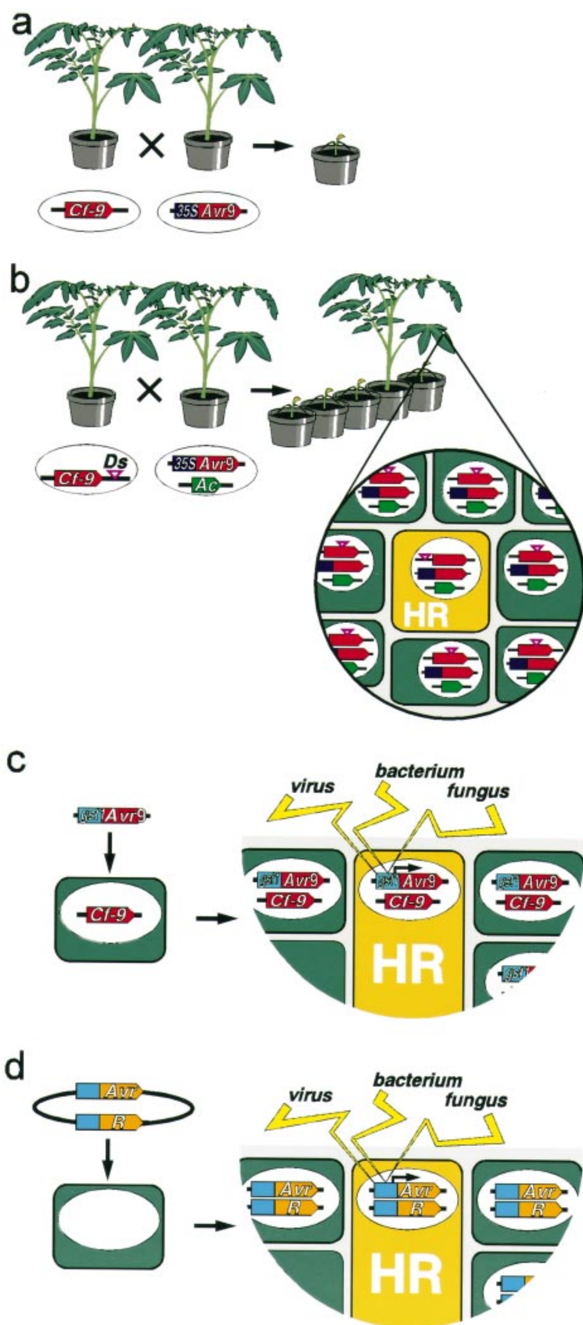

Figure 5 Exploitation of R and matching Avr genes in molecular resistance breeding. Combination of resistance gene Cf-9 and constitutively produced AVR9 (35S:Avr9) in one plant is lethal (*a*). If in most parts of the tissue, the Cf-9 gene is inactivated by a transposon (Ds), normal healthy plants develop. However, somatic excision of the transposon due to the presence of Ac results in localized restoration of the Cf-9 gene, leading to HR and systemic activation of defense responses (*b*). The two-component sensor system employs the Avr9 gene placed under control of a pathogen-inducible promoter fragment (gst1) in a Cf-9 background. Pathogen attack results in localized transcriptional activation of Avr9, resulting in HR and resistance (*c*). If both the R and matching Avr genes have been cloned, they can be combined in a double construct, which can be introduced in any species that retains a functional signal transduction pathway leading to HR. Either one or both of the genes can be placed under control of a pathogen-inducible promoter (*d*). See text for further details.

"leaky" expression patterns. Simultaneous expression, resulting in localized HR, should occur only upon attack by a pathogen.

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