The Chitin-Binding *Cladosporium fulvum* Effector Protein Avr4 Is a Virulence Factor

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The biotrophic fungal pathogen *Cladosporium fulvum* (syn. *Passalora fulva*) is the causal agent of tomato leaf mold. The Avr4 protein belongs to a set of effectors that is secreted by *C. fulvum* during infection and is thought to play a role in pathogen virulence. Previous studies have shown that Avr4 binds to chitin present in fungal cell walls and that, through this binding, Avr4 can protect these cell walls against hydrolysis by plant chitinases. In this study, we demonstrate that Avr4 expression in *Arabidopsis* results in increased virulence of several fungal pathogens with exposed chitin in their cell walls, whereas the virulence of a bacterium and an oomycete remained unaltered. Heterologous expression of Avr4 in tomato increased the virulence of *Fusarium oxysporum* f. sp. *lycopersici*. Through tomato GeneChip analyses, we demonstrate that Avr4 expression in tomato results in the induced expression of only a few genes. Finally, we demonstrate that silencing of the Avr4 gene in *C. fulvum* decreases its virulence on tomato. This is the first report on the intrinsic function of a fungal avirulence protein that has a counter-defensive activity required for full virulence of the pathogen.

Additional keywords: hypersensitive response, resistance gene.

The biotrophic fungal pathogen *Cladosporium fulvum* (syn. *Passalora fulva*) is the causal agent of tomato leaf mold (de Wit 1992, Thomma et al. 2005). Colonization of leaves by the fungus is restricted to the apoplast, and no haustoria or other feeding structures are produced (Bond 1938; de Wit 1977; Lazarovits and Higgins 1976). Therefore, all molecular components of both plant and fungus that are involved in the interaction are present in the apoplast. Eight fungal proteins that are secreted by *C. fulvum* during infection have been characterized and the corresponding genes have been cloned (Joosten et al. 1994; Laugé et al. 2000; Luderer et al. 2002; Thomma et al. 2005; van den Ackerveken et al. 1993; van Kan et al. 1991; Westerink et al. 2004). This set consists of four race-specific avirulence proteins (Avrs) (Avr2, Avr4, Avr4E, and Avr9), and four extracellular proteins (Ecp1, Ecp2, Ecp4, and Ecp5) that, in contrast to the Avrs, show much less polymorphism because the cognate *Cf-Ecp* genes have not, or rarely have been used in commercial tomato lines (Thomma et al. 2005; I. Stergiopoulos

and P. J. G. M. de Wit, *unpublished data*). Despite a number of common features shared by all *C. fulvum* effector proteins, such as their small size and even number of cysteine residues, they display no sequence similarity to each other or any other protein sequence present in public databases.

Resistance in tomato against *C. fulvum* is governed in a gene-for-gene manner as initially proposed by Flor (1942) and Oort (1944) for the flax–*Melampsora lini* and the wheat–*Ustilago tritici* pathosystems, respectively. It is conferred by dominant *C. fulvum* (*Cf*) resistance genes that activate a defense cascade eventually leading to a hypersensitive response (HR) and host immunity (Kruijt et al. 2005). The fungus can evade this immunity if it lacks the cognate *Avr* gene or carries mutant *avr* alleles that are no longer recognized by the plant. For all four *Avr* genes, the cognate *Cf* resistance genes have been cloned (Dixon et al. 1996, 1998; Jones et al. 1994; Takken et al. 1998; Thomas et al. 1997), while *Cf* loci that confer recognition of the *Ecps* also have been described (de Kock et al. 2005; Laugé et al. 1998). Although the *Lycopersicon* genus has developed *Cf* proteins to mediate recognition of *C. fulvum* effector proteins, the effector genes are maintained within the fungal population. Furthermore, the effector genes are highly expressed in planta during colonization of the host (de Wit 1992; Joosten et al. 1997; Thomma et al. 2006; van den Ackerveken et al. 1994; van Kan et al. 1991). Altogether, these observations suggest that *C. fulvum* effectors play a role in pathogen virulence (Thomma et al. 2005).

At present, the intrinsic functions of a growing number of pathogen effectors, mostly those of bacterial origin, are being uncovered. However, the intrinsic functions of only a few fungal and oomycetous plant pathogens have been described. For *C. fulvum*, it has been shown that the secreted effector protein Avr2 inhibits the extracellular tomato cysteine protease Rcr3 which is guarded by the tomato extracellular, membrane-anchored resistance protein Cf-2 in resistant tomato cultivars (Rooney et al. 2005). When Avr2 binds and inhibits Rcr3, this interaction somehow is perceived by the Cf-2 protein, resulting in an HR and resistance (Rooney et al. 2005). Several secreted effectors from the oomycetous pathogens *Phytophthora infestans* have been identified that also display protease-inhibitory activity, and may thus suppress host defense responses (Tian et al. 2004, 2005, 2007). Nevertheless, it remains to be demonstrated that these effectors indeed are required for full pathogen virulence. In contrast, it recently was shown that two avirulence proteins from the barley powdery mildew fungus increase fungal infectivity on susceptible host plant cells; however, their intrinsic functions remain to be discovered (Ridout et al. 2006). Similarly, from *C. fulvum*, two novel in planta-secreted proteins recently have been

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identified that are required for full virulence; however, for these proteins as well, their intrinsic functions are not known yet (M. D. Bolton and B. P. H. J. Thomma *unpublished results*).

In addition to the *C. fulvum* Avr2 effector described above, a putative intrinsic function has been ascribed to the Avr4 effector. Initially, in vitro experiments showed that this effector specifically binds to fungal cell walls, but not to those of tomato (Westerink et al. 2002). Subsequently, based on structural analysis, Avr4 was found to harbor an invertebrate chitin-binding domain (van den Burg et al. 2003, 2004). Chitin is a major component of fungal cell walls and is targeted by plant chitinases as part of the plant's defense response (Grison et al. 1996; Jongedijk et al. 1995; Mauch et al. 1988; Schlumbaum et al. 1986; Sela-Buurlage et al. 1993). Avr4 was found to bind specifically to chitin present in fungal cell walls in planta (van den Burg et al. 2006) and it has been suggested that, through this binding, Avr4 can protect fungal hyphae against hydrolysis by plant chitinases. This has been demonstrated in vitro for the fungal species *Fusarium solani* and *Trichoderma viride* (van den Burg et al. 2006). Chitin in the cell walls of in vitro-grown *C. fulvum* was found to be inaccessible to either chitinases or Avr4 due to the presence of a matrix of glucans and proteins covering the chitin present in cell walls of *C. fulvum* grown in vitro (de Wit and Kodde 1981; de Wit and Roseboom 1980). However, it was demonstrated that Avr4 does bind to the hyphae of *C. fulvum* present in the apoplast of infected tomato plants. This suggests that Avr4 may protect *C. fulvum* hyphae from the activity of chitinases that accumulate in the tomato apoplast during infection (van den Burg et al. 2006; Wubben et al. 1993). Interestingly, natural strains of *C. fulvum* have been identified that produce Avr4 isoforms which are degraded by proteases in the tomato apoplast but have retained their chitin-binding ability (van den Burg et al. 2003). In this way, Cf-4-mediated recognition of mutant isoforms of Avr4 by the Cf-4 protein in the cell wall of tomato is evaded, but possibly without loss of the intrinsic function, suggesting that both native and mutant forms of Avr4 contribute to fitness of *C. fulvum* (van den Burg et al. 2003). However, experimental proof for Avr4 being a genuine virulence factor for *C. fulvum* is still lacking.

In this study, we demonstrate that Avr4, when heterologously expressed in either tomato or *Arabidopsis*, contributes to the virulence of several fungal pathogens of both host plants. In addition, we show that silencing of the *Avr4* gene in *C. fulvum* decreases the virulence of this pathogen on its host plant tomato. To our knowledge, this is the first report of a fungal avirulence protein in which intrinsic activity can be implicated directly in full fungal virulence on its host plant.

Table 1. Differentially expressed genes in 4-week-old Avr4-producing transgenic tomato plants when compared with control plants as revealed by Affymetrix tomato genechips

Array fold change	Annotation
-8.21	Similar to <i>Solanum tuberosum</i> vacuolar sorting receptor protein
-4.15	Polyphenol oxidase
-3.96	Polyphenol oxidase
-2.59 ^a	Chitinase
3.02 ^b	Similar to <i>Arabidopsis thaliana</i> RNA-binding protein-like
3.42 ^b	Induced in tomato root during or after fruit set
4.83 ^b	Hypothetical protein

^a This differential could not be confirmed by quantitative real-time polymerase chain reaction analysis.

^b These genes are also differentially regulated in transgenic tomato plants that produce the *Cladosporium fulvum* effector protein Avr9.

RESULTS

Heterologous expression of Avr4 in tomato does not promote virulence of a natural Avr4 nonproducing strain of *C. fulvum*.

Strain 38 of *C. fulvum* (Bailey and Kerr 1964) has been described not to produce functional Avr4 protein (Joosten et al. 1997). This strain carries a single nucleotide deletion in the *Avr4* open reading frame, which results in a frame shift and the production of a truncated Avr4 protein of only 13 amino acids, whereas the wild-type, mature Avr4 consists of 86 amino acids (Joosten et al. 1997). We used this strain to investigate the role of Avr4 in *C. fulvum* virulence. Previously, transgenic Money-Maker-Cf-0 tomato plants (MM-Cf-0) constitutively producing Avr4 that is secreted into the apoplast have been generated and characterized (Thomas et al. 1997). Using this transgenic tomato line, we evaluated whether the presence of apoplastic Avr4 produced by the host is able to promote virulence of *C. fulvum* strain 38. Four-week-old transgenic Avr4-producing tomato plants and the parental MM-Cf-0 line were inoculated with conidia of *C. fulvum* strain 38, and disease progression was monitored visually up to 3 weeks after inoculation. No difference in disease progression could be observed on the two different tomato lines (results not shown). For both genotypes, appearance of the first symptoms, emergence of conidiophores from the stomata, and the timing and the level of sporulation were not significantly different. Subsequent measurement of the *C. fulvum* biomass with real-time polymerase chain reaction (PCR) confirmed similar growth of *C. fulvum* strain 38 on both tomato genotypes (results not shown).

Apoplastic expression of Avr4 in tomato does not cause significant changes in transcription.

Because no obvious macroscopically visible phenotypic differences could be observed, we subsequently analyzed whether the presence of Avr4 in the apoplast of tomato leaves caused significant transcriptional changes in the plant. To this end, we used a custom-designed Affymetrix tomato GeneChip array (Syngenta Biotechnology, Inc., Research Triangle Park, NC, U.S.A.) that contains probe sets representing 22,721 tomato gene transcripts. Recently, cDNA-amplified fragment length polymorphism (AFLP) analysis revealed that expression of *Avr4* in tomato plants carrying the cognate resistance gene *Cf-4* leads to extensive transcriptional reprogramming during synchronously induced host cell death (Gabriëls et al. 2006). Expression profiles from leaves of 4-week-old Avr4-producing transgenic tomato lines were compared with those of leaves of the parental MM-Cf-0 line. In total, only seven differentially expressed genes were identified that showed at least a twofold change in expression level between the two tomato lines (Table 1). This differential expression was confirmed with real-time PCR analy-

Fig. 1. Detection of Avr4 in apoplastic fluid isolated from transgenic *Arabidopsis* plants. Western analyses of apoplastic fluids isolated from three independently transformed *Arabidopsis* lines that constitutively express the *Cladosporium fulvum* effector gene *Avr4* and from the parental Col-0 line, using an Avr4-specific polyclonal antibody. The Avr4-specific signal at the expected molecular weight (approximately 9 kDa, indicated by arrow) is observed only in the transgenic *Arabidopsis* lines expressing *Avr4*.

sis for all genes except for the chitinase gene (data not shown). Interestingly, of the six remaining differentially expressed genes, three also were upregulated in transgenic tomato lines expressing the *C. fulvum* Avr9 gene (data not shown). Apart from the presence of a high number of cysteine residues that all are involved in disulfide bridges, there is no homology between Avr4 and Avr9. Therefore, it can be concluded that, so far, only three tomato genes display Avr4-specific downregulated expression; one gene encodes a vacuolar sorting receptor protein and the other two encode polyphenol oxidases (Table 1). These results show that the presence of Avr4 in the apoplast of tomato causes remarkably small effects on the plant transcriptome in the absence of the cognate *Cf-4* resistance gene.

Avr4-producing *Arabidopsis* plants are more susceptible to several fungal pathogens.

The model plant *Arabidopsis thaliana* is well characterized with respect to pathogen defense responses (Thomma et al. 2001). To further investigate the activity of Avr4 in planta, transgenic *Arabidopsis* plants that constitutively produce Avr4 were generated. A binary construct with cDNA encoding the mature Avr4 protein fused to the coding region for the tobacco PR1a signal sequence for extracellular targeting and driven by the constitutive CaMV 35S promoter was transformed into the *Arabidopsis* ecotype Col-0 (Clough and Bent 1998). Several independent homozygous single-integration lines were obtained and screened for Avr4 production by Western analyses (Fig. 1) of total protein extracts with Avr4-specific polyclonal antibodies (van Esse et al. 2006). The three lines that displayed the highest levels of Avr4 production were chosen for further

analysis. None of the Avr4-producing *Arabidopsis* plants showed any macroscopically visible phenotypic anomalies when grown under standard greenhouse conditions.

C. fulvum is not a pathogen of *Arabidopsis*; therefore, we used typical *Arabidopsis* pathogens to challenge the Avr4-producing *Arabidopsis* transgenes (Thomma et al. 1998, 2000, 2001). We found that 4-week-old greenhouse-grown control and Avr4-producing plants inoculated with conidia of the necrotrophic fungal pathogen *Botrytis cinerea* showed a clear difference in disease progression. Avr4-producing *Arabidopsis* lines showed significantly stronger disease symptoms compared with both the parental control line and Avr9-producing *Arabidopsis* (Fig. 2). On the Avr4-producing plants, lesions developed faster, showed a larger diameter, and displayed chlorotic halos that were contained in a later stage. A similar increase in disease susceptibility was observed for Avr4-producing *Arabidopsis* plants when inoculated with the necrotrophic fungus *Plectosphaerella cucumerina* (Fig. 2), although assays with the necrotrophic fungus *Alternaria brassicicola* did not convincingly reveal increased disease susceptibility of Avr4-producing plants. However, in contrast to *B. cinerea* and *Plectosphaerella cucumerina*, *A. brassicicola* behaves as an incompatible pathogen on wild-type *Arabidopsis* plants (Thomma 2003; Thomma et al. 1998), which may be due to host factors other than chitinases. To investigate whether production of Avr4 also increases virulence of pathogens other than fungi, the symptoms caused by the hemibiotrophic oomycete *Phytophthora brassicae* and the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 also were analyzed. Interestingly, no increase in virulence was observed for either of these two pathogens (Fig. 2).

Fig. 2. Avr4-producing *Arabidopsis* is more susceptible to several fungal pathogens. Typical symptoms caused by *Botrytis cinerea*, *Plectosphaerella cucumerina*, *Phytophthora brassicae*, and *Pseudomonas syringae* on 4-week-old plants of three independent Avr4-producing *Arabidopsis* lines at 4 days postinoculation. Disease progression by *B. cinerea* and *Plectosphaerella cucumerina* is faster on Avr4-producing *Arabidopsis* than on the parental Col-0 line and Avr9-producing *Arabidopsis*. No differences in disease progression by the oomycetous pathogen *Phytophthora brassicae* or the bacterial pathogen *Pseudomonas syringae* are observed on the same set of *Arabidopsis* lines.

For *P. syringae*, we measured the bacterial colonization using real-time PCR quantification (Brouwer et al. 2003) confirming the lack of significant differences between wild-type and Avr4-producing *Arabidopsis* genotypes (results not shown). Taken together, our data suggest that, on *Arabidopsis*, Avr4 promotes the virulence of fungal pathogens but not of oomycetous or bacterial pathogens.

Avr4 protects various fungi against chitinases in vitro.

It has been demonstrated previously that Avr4 protects *F. solani* f. sp. *phaseoli* and *T. viride* against the deleterious effects of plant chitinases in vitro (van den Burg et al. 2006). To test whether Avr4 also is able to protect the *Arabidopsis* pathogens *B. cinerea* and *Plectosphaerella cucumerina* against chitinase activity, we performed similar in vitro assays for these two fungi, using *C. fulvum* as a control. Conidia were germinated, incubated with *Pichia pastoris*-produced Avr4, and subsequently treated with a crude extract of tomato leaves contain-

ing intracellular, basic chitinases. Growth of *Plectosphaerella cucumerina* clearly was inhibited by the hydrolytic enzymes present in this extract, and Avr4 was able to protect the fungus against the deleterious effects in a dose-dependent manner (Fig. 3). However, growth of *B. cinerea* was not inhibited by the treatment with chitinases, thus rendering it impossible to test whether Avr4 can protect this fungus against the activity of tomato chitinases. Similarly, it previously has been shown that in vitro growth of *C. fulvum* is not inhibited by chitinases, and that Avr4 does not bind to hyphae of in vitro-grown *C. fulvum* (Joosten et al. 1995; van den Burg et al. 2006). In contrast, during colonization, in planta chitin in the hyphae of this fungus appeared to be highly accessible (van den Burg et al. 2006). A similar situation may be true for *B. cinerea*.

Heterologous expression of Avr4 in tomato enhances susceptibility to *F. oxysporum* f. sp. *lycopersici*.

Species of the genus *Fusarium* generally are known to be sensitive to chitinases. Therefore, we investigated whether transgenic Avr4-producing tomato plants show increased susceptibility to *F. oxysporum* f. sp. *lycopersici*. After inoculation

Fig. 3. Avr4 protects *Arabidopsis* and tomato fungal pathogens against chitinases in vitro. Micrographs of *Plectosphaerella cucumerina*, *Cladosporium fulvum*, and *Fusarium oxysporum* f. sp. *lycopersici* taken 24 h after addition of a crude extract of tomato leaves containing intracellular, basic chitinases to the culture medium. Growth of *P. cucumerina* and *F. oxysporum* is inhibited in the presence of chitinases (control), but is sustained in the same medium in the presence of 50 μ M Avr4 (+ Avr4). As noted previously, *C. fulvum* is not sensitive to chitinases in vitro.

Fig. 4. Heterologous expression of Avr4 in tomato results in increased susceptibility to *Fusarium oxysporum* f. sp. *lycopersici*. Typical symptoms of disease after inoculation of 4-week-old Avr4-producing tomato plants with *F. oxysporum* f. sp. *lycopersici* at 14 days postinoculation. **A**, Mock-inoculated Avr4-producing MM-Cf-0 tomato. **B**, Mock-inoculated control tomato. **C**, *F. oxysporum*-inoculated Avr4-producing tomato. **D**, *F. oxysporum*-inoculated control MM-Cf-0 tomato. Disease symptoms are more pronounced on Avr4-producing MM-Cf-0 tomato plants when compared with MM-Cf-0 controls.

Fig. 5. Silencing of Avr4-expression in *Cladosporium fulvum* decreases its virulence on its host tomato. **A**, Quantitative real-time polymerase chain reaction (PCR) of Avr4 transcript levels during a compatible interaction with MM-Cf-0 tomato. Avr4 transcript levels are significantly reduced in four independent Avr4-silenced *C. fulvum* transformants when compared with the parental strain (control). **B**, Disease symptoms developed after inoculation of MM-Cf-0 tomato plants with the same four *C. fulvum* transformants or the parental strain (control), monitored at 11 days postinoculation. Mycelium of the *C. fulvum* parental strain reemerging from stomata on the lower side of the leaves has almost completely covered the whole leaf surface, whereas much smaller, dispersed patches of mycelium are visible on leaves of plants inoculated with the Avr4-silenced transformants of the fungus. **C**, Quantitative real-time PCR of fungal colonization by comparing *C. fulvum* actin transcript levels (as a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) at 11 days postinoculation.

of *F. oxysporum* f. sp. *lycopersici* on 4-week-old transgenic Avr4-producing tomato plants and the parental MM-Cf-0 line, disease progression was monitored. Interestingly, disease symptoms developed earlier and were more pronounced on the transgenic Avr4-producing tomato plants compared with the control, exemplified by a faster disease progression, earlier wilting, and chlorosis. In addition, inoculated Avr4-transgenic tomato plants were significantly shorter than inoculated control plants (Fig. 4). Furthermore, pre-incubation of germinated *F. oxysporum* f. sp. *lycopersici* conidia with Avr4 was found to protect the fungal hyphae against the deleterious activity of tomato chitinases (Fig. 3).

Silencing of Avr4 compromises virulence of *C. fulvum*.

Our results obtained with tomato and *Arabidopsis* plants expressing Avr4 show that Avr4 increases virulence of chitin-containing fungal pathogens. Therefore, we investigated the role of Avr4 in virulence of *C. fulvum* itself by employing Avr4-specific gene silencing. A binary vector was generated containing an inverted repeat fragment of the *Avr4* gene, driven by the *ToxA* promoter of the fungal wheat pathogen *Pyrenophora tritici-repentis* for constitutive expression (Ciuffetti et al. 1997). Using *Agrobacterium tumefaciens*-mediated transformation (M. D. Bolton and B. P. H. J. Thomma unpublished results), this construct was introduced into a race 4 strain of *C. fulvum*, race 4(2), which produces an Avr4 isoform containing a Tyr⁶⁷-to-His amino acid substitution (Joosten et al. 1997). Several Avr4 inverted repeat transformants were obtained, four of which were used for further analysis. To determine whether the introduction of the *Avr4* inverted repeat construct resulted in *Avr4* silencing, 4-week-old MM-Cf-0 tomato plants were inoculated with the four transgenic *C. fulvum* strains. The in planta expression levels of *Avr4* were determined relative to the constitutively expressed *C. fulvum* actin gene using real-time PCR. It appeared that *Avr4* expression was strongly reduced in each of the four transformants when compared with *Avr4* expression level in the parental *C. fulvum* strain (Fig. 5A). Virulence assays on MM-Cf-0 tomato plants showed that the *Avr4*-silenced transformants were significantly compromised in their ability to colonize tomato leaves when compared with the parental *C. fulvum* strain (Fig. 5B). Also, the biomass produced by the *C. fulvum* transformants was significantly lower than that produced by the parental strain, as could be shown by real-time PCR quantification of *C. fulvum* actin transcripts (Fig. 5C).

DISCUSSION

The *C. fulvum* Avr4 protein provides the first example of a fungal avirulence protein whose intrinsic biological function is demonstrated to contribute to pathogen virulence. It has been demonstrated previously that Avr4 is a lectin with chitin-binding activity that can protect fungal cell walls against the deleterious activity of plant chitinases (van den Burg et al. 2003, 2004, 2006). By generating transgenic plants that heterologously express the *C. fulvum* *Avr4* gene, we now demonstrate that the presence of apoplastic Avr4 promotes the virulence of several fungal pathogens in *Arabidopsis* and tomato. Furthermore, *Avr4* silencing in *C. fulvum* clearly results in compromised virulence, demonstrating that Avr4 is an effector protein that contributes to pathogen virulence. This observation also may comply with the current view that pathogen effector molecules are evolved mainly to counteract pathogen-associated molecular pattern (PAMP)-triggered basal immune responses in the plant (Chisholm et al. 2006; Jones and Dangl 2006). Chitin oligosaccharides are known to act as PAMPs that trigger strong host defense responses, including the accumulation of chitinases

(Ramonell et al. 2002, 2005; Shibuya and Minami 2001), and, recently, a plasma membrane receptor for chitin oligosaccharides has been identified in rice (Kaku et al. 2006). The in planta production of Avr4 by *C. fulvum* not only protects chitin in the fungal cell wall against hydrolysis by host chitinases but also may reduce the release of chitin fragments that could trigger host immune responses. In Cf-4 tomato plants, detection of Avr4 produced by *C. fulvum* results in an effector-triggered Cf-4-mediated immune response, which may be overcome by the fungus by the production of protease-sensitive Avr4 isoforms that are quickly degraded in the apoplast and, thus, are no longer recognized (Joosten et al. 1994, 1997).

Initial experiments with a natural race 4 *C. fulvum* strain that does not produce functional Avr4 (strain 38) failed to demonstrate increased virulence of this strain in transgenic Avr4-producing tomato lines. This may be explained by functional redundancy among pathogen effector molecules, implying that *C. fulvum* has additional chitin-binding effectors that are different from Avr4. Recently, a novel *C. fulvum* effector, Ecp6, has been identified that carries three lysin motifs (LysM domains) (M. D. Bolton and B. P. H. J. Thomma unpublished results). Interestingly, these motifs are found in a wide variety of proteins that are involved in oligosaccharide binding, including chitinases and the rice chitin-binding plasma membrane receptor that acts in innate immunity (Amon et al. 2000; Joris et al. 1992; Kaku et al. 2006; Limpens et al. 2003; Madsen et al. 2003; Ponting et al. 1999; Radutoiu et al. 2003). Furthermore, a glycoprotein with LysM domains has been identified in the plant-pathogenic fungus *Colletotrichum lindemuthianum* that was found to bind to fungal hyphae in planta (Perfect et al. 1998) in a way similar to that observed for Avr4 of *Cladosporium fulvum* (van den Burg et al. 2006). This all suggests that Ecp6 may be a functional, chitin-binding homologue of Avr4 (M. D. Bolton and B. P. H. J. Thomma unpublished results).

For the gene-silencing experiments, a race 4 strain of *C. fulvum* was used that produces an Avr4 isoform with a Tyr⁶⁷-to-His amino acid substitution (Joosten et al. 1997). By using the *Potato virus X* expression system, it was shown that this *avr4* allele encodes an Avr4 isoform that is still somewhat active as an elicitor in Cf-4 tomato plants. However, because this strain evades recognition in Cf-4 plants, it was suggested that this Avr4 isoform is quickly degraded upon secretion (Joosten et al. 1997). Several Avr4 isoforms have been characterized that are sensitive to proteolysis, thereby circumventing Cf-4-mediated resistance, yet retain their chitin-binding ability (van den Burg et al. 2003). Upon binding to chitin, these unstable isoforms remain stable (van den Burg et al. 2003). For our gene-silencing experiments, we decided to use a *C. fulvum* strain that produces an unstable isoform of Avr4. We anticipated that the increased turnover of an unstable Avr4 isoform compared with the wild-type Avr4 would counterbalance the effect of residual Avr4 production as a result of incomplete gene silencing. Interestingly, although this Avr4 isoform evades Cf-4 mediated recognition, it has retained its virulence function, demonstrating that natural Avr4 mutants that no longer trigger an HR in tomato still may contribute to fitness of *C. fulvum*. Nevertheless, natural *C. fulvum* strains that do not produce any functional Avr4 also exist. However, it is conceivable that the pathogenicity of a natural strain that completely lacks the production of Avr4 requires the production of a functional homologue or homologues to protect itself against the deleterious effects of plant chitinases. Based on our results, it is suggested that race 4 *C. fulvum* strains that produce Avr4 mutants that have retained chitin-binding ability do not necessarily require these homologues.

We also assessed whether Avr4 could have a direct function as host defense modulator. It has been shown previously that the progeny of a cross between MM-Cf-0 tomato plants that

express *C. fulvum* *Avr4* and MM-Cf-4 tomato plants generates viable Cf-4/*Avr4* seed that are able to germinate. However, after germination, the progeny displays lethality at the seedling stage due to the initiation of an HR (Thomas et al. 1997). This HR-initiation is temperature sensitive and can be prevented by growth of the Cf-4/*Avr4* seedlings at 33°C and high relative humidity (de Jong et al. 2002; Thomas et al. 1997). Subsequent transfer of these seedlings from 33 to 20°C leads to a synchronous and systemic initiation of the HR. A cDNA-AFLP transcriptome analysis on Cf-4/*Avr4* seedlings after transfer from 33 to 20°C has shown an extensive reprogramming of gene transcription, including many genes that are thought to act in primary metabolism and in stress signaling cascades (Gabiëls et al. 2006). Remarkably, production of *C. fulvum* *Avr4* in tomato plants that do not carry the *Cf-4* resistance gene only results in very few and rather slight transcriptional changes (Table 1). The three genes whose expression was found to be three- to fivefold induced are similarly upregulated in *Avr9*-expressing tomato plants, suggesting that their induced expression is not caused by intrinsic *Avr4* activity. The chitinase gene that was found to be slightly repressed in the array could not be confirmed by real-time PCR analyses. That leaves only three genes for which expression is specifically altered upon expression of *Avr4* and, remarkably, their expression is repressed. Two of those genes encode a polyphenol oxidase, proteins of which the exact function is still unclear (Mayer et al. 2006). In some cases, a positive correlation has been found between polyphenol oxidase activity levels and pathogen resistance (Li and Steffens 2002; Rai et al. 2006; Thipyapong et al. 2004), although it is not clear whether this relationship is causal (Mayer et al. 2006). Nevertheless, the *Avr4*-specific repression of both tomato genes is quite modest and unlikely to account for the observed enhanced pathogen susceptibility. The strongest *Avr4*-specific reduction of expression is eightfold and was observed for a gene encoding a vacuolar sorting receptor protein. These receptor proteins select proteins in the trans-Golgi for sorting to clathrin-coated vesicles and delivery to the vacuole (Kirsch et al. 1994). In this way, it has been demonstrated that seed storage proteins such as 2S albumin are delivered into the vacuoles of seed (Jolliffe et al. 2004; Shimada et al. 2003). A direct link between vacuolar sorting receptor proteins and defense against pathogens has not been demonstrated, although plant vacuoles are rich in antimicrobial components that are likely to be transported through a similar mechanism. In addition, also for seed storage proteins like 2S albumins, in vitro antimicrobial activity has been demonstrated (Terras et al. 1992, 1993). In our study, it has not been investigated whether the tomato genes, the expression of which is specifically altered upon expression of *Avr4* play a role in resistance against *C. fulvum*, or whether expression of their *Arabidopsis* homologues is similarly altered upon *Avr4*-expression.

Altogether, the chitin-binding activity is likely to be the sole intrinsic function of the *Avr4* effector protein, which is supported by the observation that the virulence of pathogens that do not contain chitin in their cell walls, such as the bacterium *Pseudomonas syringae* and the oomycete *Phytophthora brassicae*, is not enhanced in *Avr4*-producing plants.

In accordance with the “guard hypothesis,” resistant plants recognize cognate pathogen avirulence proteins indirectly by sensing the manipulation of their host targets (guardees) by R proteins (guards), which subsequently triggers an immune response (Axtell and Staskawicz 2003; Mackey et al. 2003; Rooney et al. 2005). Such an indirect interaction has been proposed for *C. fulvum* *Avr9* (Luderer et al. 2001) and, indeed, has been demonstrated for *C. fulvum* *Avr2*, that targets the tomato cysteine protease Rcr3 which is guarded by the Cf-2 protein (Rooney et al. 2005). However, examples of a direct

interaction between plant resistance proteins and pathogen avirulence proteins also have been demonstrated in the pathosystems *Magnaporthe grisea*–rice (Jia et al. 2000), *Melampsora lini*–flax (Dodds et al. 2006), *Ralstonia solanacearum*–*Arabidopsis* (Deslandes et al. 2003), and *Tobacco mosaic virus*–tobacco (Ueda et al. 2006). *Avr4* presents a strong candidate that may be recognized directly by the tomato Cf-4 resistance protein because we, and others (Westerink et al. 2002), have not been able to detect a target in the host plant tomato that could possibly act as a guardee. Future experiments will show whether *Avr4* indeed interacts with the Cf-4 protein in planta.

MATERIALS AND METHODS

Cultivation of microorganisms and plants.

C. fulvum strain 38 (Bailey and Kerr 1964) and *F. oxysporum* f. sp. *lycopersici* (provided by B. Lievens, Scientia Terrae, Belgium) were cultured at room temperature on half-strength potato dextrose agar (Oxoid, Basingstoke, England) supplemented with technical agar (Oxoid) at 7 g/liter. *Alternaria brassicicola*, *B. cinerea* (Brouwer et al. 2003), and *Plectosphaerella cucumerina* (Thomma et al. 2000) were cultured at room temperature on malt extract agar (Oxoid). *Phytophthora brassicae* isolate HH (provided by F. Govers, Wageningen University, The Netherlands) were grown on V8 juice (Campbell Soups, Camden, NJ, U.S.A.) agar (Erwin and Ribeiro 1996) in the dark at 18°C. *Pseudomonas syringae* pv. *tomato* DC3000 was cultured on King’s B medium containing rifampicin at 200 µg/ml.

All tomato plants were grown in soil under standard greenhouse conditions: 21 and 19°C during the day and night periods of 16 and 8 h, respectively; 70% relative humidity (RH); and 100 W/m² supplemental light when the intensity dropped below 150 W/m². *Arabidopsis* plants were grown in soil under similar greenhouse conditions, with 21 and 19°C during the day and night periods of 16 and 8 h, respectively; 60% RH; and 100 W/m² supplemental light when the intensity dropped below 150 W/m².

Plant inoculations.

Inoculation of *Arabidopsis* plants with *B. cinerea*, *Plectosphaerella cucumerina*, *Phytophthora brassicae*, and *Pseudomonas syringae* were performed on 4-week-old soil-grown plants. For *B. cinerea*, plants were inoculated by placing two 4-µl drops of a conidial suspension (5×10^5 conidia/ml) in potato dextrose broth (Difco, Detroit) at 12 g/liter on each leaf. Inoculation with *A. brassicicola* and *Plectosphaerella cucumerina* was performed in a similar way, using an aqueous suspension containing 5×10^5 conidia/ml. For all pathogens, plants were incubated at 20°C and 100% RH and a light and dark regime of 16 and 8 h, respectively. Disease progression was scored at 4 days postinoculation.

Inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 was performed by spray inoculation of a bacterial suspension of 5×10^8 CFU/ml in 10 mM MgCl₂ and 0.05% Silwet L-77 (Lehle Seeds, Round Rock, TX, U.S.A.) onto the leaves until “droplet run-off.” Plants were incubated at 100% RH for 1 h, followed by incubation at 20°C, 60% RH, and a 16-and-8-h light-and-dark regime. Disease progression was scored at 4 days postinoculation.

Inoculation with *Phytophthora brassicae* was performed by placing 5-mm-diameter plugs of a 2-week-old *P. brassicae* agar plate culture onto *Arabidopsis* leaves. Subsequently, the plants were incubated at 16°C, 100% RH, and a 16-and-8-h light-and-dark regime.

Inoculation with *C. fulvum* was performed as previously described (de Wit 1977). Briefly, 5-week-old soil-grown tomato

plants were inoculated by spraying 5 ml of conidial suspension (1×10^6 conidia/ml) onto the lower surface of the leaves. Subsequently, plants were kept at 100% RH for 48 h under a transparent plastic cover, after which they were incubated at standard greenhouse conditions of 16 and 8 h of light and dark, respectively, and 70% RH. Disease progression was monitored for 20 days postinoculation.

For inoculation with *F. oxysporum* f. sp. *lycopersici*, 3-week-old soil-grown tomato plants were uprooted and inoculated by dipping the roots in a conidial suspension (1×10^7 conidia/ml) in potato dextrose broth (Oxoid) at 12 g/liter. After replanting in soil, plants were incubated at standard greenhouse conditions of 16 and 8 h of light and dark, respectively, and 70% RH. Disease progression was monitored for 20 days postinoculation.

Microarray sample preparation and analyses.

For each experiment, independently repeated three times, three transgenic tomato plants and three MM-Cf-0 tomato plants were grown for 4 weeks under standard greenhouse conditions. For each plant set, all third and fourth compound leaves containing five to seven leaflets were harvested, pooled, and flash frozen in liquid nitrogen. For RNA extraction, the frozen leaves were crushed using a spoon and approximately 100 mg of the crushed material was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). After phase separation, isopropanol was added to the aqueous phase, which subsequently was further purified with the NucleoSpin RNA plant kit (Macherey-Nagel GmbH, Düren, Germany). In this way, from plants grown in three independent experiments, total RNA was obtained that was used for hybridization onto six individual custom-designed Affymetrix tomato GeneChip arrays (made available through Syngenta Biotechnology) which contained probe sets to interrogate 22,721 genes. Probe preparations and GeneChip hybridizations were carried out at ServiceXS (Leiden, The Netherlands). Microarray data sets were equilibrated and investigated with Rosetta Resolver Software (Rosetta Biosoftware, Seattle, WA, U.S.A.), which uses a proprietary algorithm error model to calculate significantly up- or downregulated sequences (Weng et al. 2006). We only considered differential signals with *P* values below 0.01 and ratios larger than two as significant.

For confirmation of differential gene expression by quantitative real-time PCR, a similar experiment was repeated for a fourth time. RNA was isolated as described. The obtained total RNA was used as a template for cDNA synthesis using an oligo(dT) primer and the SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer's guidelines. Second-strand synthesis was performed with RNaseH and DNA Polymerase I according to the manufacturer's guidelines (Promega Corp., Madison, WI, U.S.A.). Primer sequences were designed based on array probe sequences (Table 2). Using tomato actin as an internal standard, quantitative real-time PCR was conducted with an ABI7300 PCR machine (Applied Biosystems, Foster City, CA, U.S.A.) in combination with the qPCR Core kit for SYBR Green I (Eurogentec, Seraing, Belgium).

Generation of transgenic

Avr4- and Avr9-producing *Arabidopsis* plants.

For in planta production of Avr4 and Avr9, the binary vectors pAvr4 (van der Hoorn et al. 2000) and pMOG978 (Honée et al. 1998), respectively, were used. These vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformants were selected on LB medium containing kanamycin at 50 µg/ml and rifampicin at 25 µg/ml. *Arabidopsis* transformants were generated via the floral-dip method (Clough and Bent 1998). First-generation transformants were selected on 50 µM kanamycin and subsequently transferred to soil. Several independent homozygous single insertion lines were selected, and homozygous T₃ lines were used for inoculations.

Western blot analyses.

Total leaf extracts were prepared by homogenizing leaf material in demineralized water and, subsequently, denatured by boiling for 5 min in an equal volume of denaturing solution (6.25 µM Tris-HCl, pH 6.8; 2% [wt/vol] sodium dodecyl sulfate; 10% [wt/vol] glycerol; 5% [vol/vol] β-mercaptoethanol; and 0.001% [wt/vol] bromophenol blue). Protein samples were separated on 16% Tricine gels and subsequently transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, U.S.A.) by electroblotting. The resulting blots were incubated overnight at 4°C in blocking buffer (1× phosphate-buffered sa-

Table 2. Primers used in this study

Primer name	Sequence (5'–3') ^a	Description
oligo-dT	TTGGATCCTCGAGTTTTTTTTTTTTTTTTT	Poly-T (<i>NcoI</i> and <i>SacI</i>)
Nco-Avr4-F	TTCCATGGATGCACTACACAACCCTCCT	<i>Avr4</i> inverted repeat (<i>NcoI</i>)
Avr4-EcoRI-R2	GAATTCATAGCCAGGATGTCCAACCGAC	<i>Avr4</i> inverted repeat (<i>EcoRI</i>)
Avr4-NotI-R2	CGCGCCGATAGCCAGGATGTCCAACCGAC	<i>Avr4</i> inverted repeat (<i>NotI</i>)
Avr4-(RT)-F	ACTCTCCTAGTCGGCACAG	<i>Cladosporium fulvum</i> <i>Avr4</i>
Avr4-(RT)-R	CAATAGCCAGGATGTCCAAC	<i>C. fulvum</i> <i>Avr4</i>
CF-GAPDH-F	GGAAACCGGAACCGTTCAG	<i>C. fulvum</i> actin
CF-GADPH-R	TGTTAGTGATCCCTTGTCATCCAA	<i>C. fulvum</i> actin
CF-Act	CATCGGCAACGAGCGATT	Tomato actin
CF-Act	TGGTACCACCAGACATGACAATG	Tomato actin
Array1-F	GCATGCCAGGATACTTTCCGTGGTAGAG	Vacuolar sorting protein
Array1-R	CATCGCAATGCTCCAGATGGTTTCAC	Vacuolar sorting protein
Array2-F	ACCTGATCTCAAATCTTGTTGGTTGCC	Polyphenol oxidase
Array2-R	CGGAGTTTCGTTCATAGGAGGGAACCTGTAG	Polyphenol oxidase
Array3-F	CAGATGAGCTTGATAAGGCGGAGTTTGC	Polyphenol oxidase
Array3-R	CCTCCAACAGTTTCAGTTATCGCCAGC	Polyphenol oxidase
Array4-F	TGAAGAATCGAAACGATGATAGATGTCCTG	Chitinase
Array4-R	CGCGCAGTATCATACCGGTAGTAC	Chitinase
Array5-F	ATGTCAGAGGAGATTGTTTCAGGTTGTTGG	RNA-binding protein-like
Array5-R	GGAGAAACCGTTCAGGAGAATGTGGC	RNA-binding protein-like
Array6-F	GGTATCCAAATACATCGTGGTCCTGATGG	Fruit set-induced
Array6-R	GGTTCTGCTCCTGGGTTGGCATTTTC	Fruit set-induced
Array7-F	CTTTGGACTAACGGAACATTTATGTCAGCG	Hypothetical protein
Array7-R	GCGCAGTAGCCAACCAACCTGAC	Hypothetical protein

^a Restriction sites are indicated in bold.

line [PBS], pH 7.3; 3% [wt/vol] bovine serum albumen [BSA]; and 0.1% [wt/vol] Tween 20). Subsequently, blots were incubated for 2 h at room temperature with 10 µl of primary (rabbit) antibody diluted in 10 ml of antibody buffer (1× PBS, pH 7.3; 0.3% [wt/vol] BSA; and 0.1% [vol/vol] Tween 20). After washing in antibody buffer, blots were incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated antibody. After washing, the HRP-conjugate was activated (SuperSignal, Pierce, Rockford, IL, U.S.A.) and detected on film (X-OMAT LS, Eastman Kodak Company, Rochester, NY, U.S.A.). Polyclonal antibodies raised against Avr4 were produced by immunization of rabbits according to standard procedures (Eurogentec) (van Esse et al. 2006). For immunization, enterokinase-treated (for affinity-tag removal), *Pichia pastoris*-produced His₆-FLAG-tagged Avr4 (van Esse et al. 2006) was used.

Isolation of intracellular chitinases from tomato.

Isolation of tomato chitinases was performed essentially as described (Joosten et al. 1990, 1995). A total protein extract was prepared from 500 g of fresh tomato leaves and soluble proteins were subjected to gel filtration with a Sephadex G-50 column (GE Healthcare, Chalfont St. Giles, U.K.), at a flow rate of 10 ml/h, and 14 fractions of 15 ml each were collected and dialyzed against demineralized water and subsequently freeze dried. Each of the freeze-dried fractions then was dissolved in 2 ml of demineralized water and filter sterilized. Subsequently, the fractions were screened for antifungal activity by challenging 50 µl of an overnight liquid culture of 100 conidia/ml of *T. viride* with 40 µl of the individual fractions (van den Burg et al. 2006).

In vitro fungal growth assays.

Approximately 10³ conidia (for *F. oxysporum* f. sp. *lycopersici*, *Plectosphaerella cucumerina*, and *C. fulvum*) or 50 conidia (for *B. cinerea*) were incubated overnight at room temperature in 50 µl of potato dextrose broth in 96-well microtiter plates. Subsequently, Avr4 protein was added to the conidial suspensions at final concentrations of 0, 0.5, 5, and 50 µM, respectively. After a 2-h incubation period, 40 µl of extract containing the tomato chitinases was added. Fungal growth was assayed microscopically after 24 h of incubation at 22°C.

Construction of plasmids

for silencing of Avr4-expression in *C. fulvum*.

A fragment of the Avr4-coding sequence was amplified using cDNA from a compatible interaction between *C. fulvum* and tomato as template. Primer sequences are shown in Table 2. Construction of the binary vector containing an inverted repeat fragment of the Avr4 gene was performed as described, and *A. tumefaciens*-mediated transformation of *C. fulvum* was performed as described (M. D. Bolton and B. P. H. J. Thomma unpublished results).

Quantification of Avr4 expression levels and fungal biomass.

C. fulvum was inoculated onto 5-week-old MM-Cf-0 tomato plants as described above. In each of three independently repeated experiments, leaf material was harvested from inoculated MM-Cf-0 plants at 0, 3, 7, 11, and 16 days postinoculation, flash frozen in liquid nitrogen, and stored at -80°C until used for RNA analysis. Leaf samples consisted of three leaflets obtained from the second, third, and fourth compound leaves of two tomato plants. Total RNA was isolated from infected leaf material using the RNeasy kit (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer's instructions. Total RNA was used for cDNA synthesis using an oligo(dT) primer

and the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was conducted with primers given in Table 2, and using an ABI7300 PCR machine (Applied Biosystems) in combination with the qPCR Core kit for SYBR Green I (Eurogentec). Real-time PCR conditions consisted of an initial 95°C denaturation step for 10 min followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 40 cycles, and were analyzed on the 7300 System SDS software (Applied Biosystems). To check for contamination with genomic DNA, real-time PCR also was carried out on RNA without the addition of reverse transcriptase.

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LITERATURE CITED

- Amon, P., Haas, E., and Sumper, M. 1998. The sex-inducing pheromone and wounding trigger the same set of genes in the multicellular green alga *Volvox*. *Plant Cell* 10:781-789.
- Axtell, M. J., and Staskawicz, B. J. 2003. Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* 112:369-377.
- Bailey, D. L., and Kerr, E. A. 1964. *Cladosporium fulvum* race 10 and resistance to it in tomato. *Can. J. Bot.* 42:1555-1558.
- Bond, T. E. T. 1938. Infection experiments with *Cladosporium fulvum* Cooke and related species. *Ann. Appl. Biol.* 25:277-307.
- Brouwer, M., Lievens, B., Van Hemelrijck, W., Van den Ackerveken, G., Cammue, B. P. A., and Thomma, B. P. H. J. 2003. Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 228:241-248.
- Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. 2006. Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* 124:803-814.
- Ciuffetti, L. M., Tuori, R. P., and Gaventa, J. M. 1997. A single gene encodes a selective toxin causal to the development of tan spot of wheat. *Plant Cell* 9:135-144.
- Clough, S. J., and Bent, A. F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735-743.
- de Jong, C. F., Takken, F. L. W., Cai, X., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2002. Attenuation of Cf-mediated defense responses at elevated temperatures correlates with a decrease in elicitor-binding sites. *Mol. Plant-Microbe Interact.* 15:1040-1049.
- de Kock, M. J. D., Brandwagt, B. F., Bonnema, G., de Wit, P. J. G. M., and Lindhout, P. 2005. The tomato Orion locus comprises a unique class of *Hcr9* genes. *Mol. Breed.* 15:409-422.
- de Wit, P. J. G. M. 1977. Light and scanning-electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum*. *Neth. J. Plant Pathol.* 83:109-122.
- de Wit, P. J. G. M. 1992. Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. *Annu. Rev. Phytopathol.* 30:391-418.
- de Wit, P. J. G. M., and Kodde, E. 1981. Further characterization and cultivar specificity of glycoprotein elicitors from culture filtrates and cell walls of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Physiol. Plant Pathol.* 18:298-314.
- de Wit, P. J. G. M., and Roseboom, P. H. M. 1980. Isolation, partial characterization and specificity of glycoprotein elicitors from culture filtrates, mycelium and cell walls of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Physiol. Plant Pathol.* 16:391-408.

- Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., and Marco, Y. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 100:8024-8029.
- Dixon, M. S., Jones, D. A., Keddle, J. S., Thomas, C. M., Harrison, K., and Jones, J. D. G. 1996. The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. *Cell* 84:451-459.
- Dixon, M. S., Hatzixanthis, K., Jones, D. A., Harrison, K., and Jones, J. D. G. 1998. The tomato *Cf-5* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. *Plant Cell* 10:1915-1925.
- Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., The, T., Wang, C. I., Ayliffe, M. A., Kobe, B., and Ellis, J. G. 2006. Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 103:8888-8893.
- Erwin, D., and Ribeiro, O. K. 1996. *Phytophthora Diseases Worldwide*. American Phytopathological Society Press, St. Paul, MN, U.S.A.
- Flor, H. H. 1942. Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* 32:653-669.
- Gabriëls, S. H. E. J., Takken, F. L. W., Vossen, J. H., de Jong, C. F., Liu, Q., Turk, S. C. H. J., Wachowski, L. K., Peters, J., Witsenboer, H. M. A., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2006. cDNA-AFLP combined with functional analysis reveals novel genes involved in the hypersensitive response. *Mol. Plant-Microbe Interact.* 19:567-576.
- Grisson, R., Grezes-Besset, B., Schneider, M., Lucante, N., Olsen, L., Leguay, J. J., and Toppan, A. 1996. Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. *Nat. Biotechnol.* 14:643-646.
- Honee, G., Buitink, J., Jabs, T., de Kloe, J., Sijbolts, F., Apotheker, M., Weide, R., Sijen, T., Stuiver, M., and de Wit, P. J. G. M. 1998. Induction of defense-related responses in *Cf9* tomato cells by the AVR9 elicitor peptide of *Cladosporium fulvum* is developmentally regulated. *Plant Physiol.* 117:809-820.
- Jia, Y., Mc Adams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:4004-4014.
- Jolliffe, N. A., Brown, J. C., Neumann, U., Vicre, M., Bachi, A., Hawes, C., Ceriotti, A., Roberts, L. M., and Frigerio, L. 2004. Transport of ricin and 2S albumin precursors to the storage vacuoles of *Ricinus communis* endosperm involves the Golgi and VSR-like receptors. *Plant J.* 39:821-833.
- Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.
- Jones, D. A., Thomas, C. M., Hammond-Kosack, K. E., Balint-Kurti, P. J., and Jones, J. D. G. 1994. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* 266:789-793.
- Jongedijk, E., Tigelaar, H. Van Roekel, J. S. C., Bres-Vloemans, S. A., Dekker, I., Van den Elzen, P. J. M., Cornelissen, B. J. C., and Melchers L. S. 1995. Synergistic activity of chitinase and beta-1,3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* 85:173-180.
- Joosten, M. H. A. J., Bergmans, C. J. B., Meulenhoff, E. J. S., Cornelissen, B. J. C., and de Wit, P. J. G. M. 1990. Purification and serological characterization of three basic 15-kilodalton pathogenesis-related proteins from tomato. *Plant Physiol.* 94:585-591.
- Joosten, M. H. A. J., Cozijnsen, T. J., and de Wit, P. J. G. M. 1994. Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature* 367:384-386.
- Joosten, M. H. A. J., Verbakel, H. M., Nettekoven, M. E., van Leeuwen, J., van der Vossen, R. T. M., and de Wit, P. J. G. M. 1995. The phytopathogenic fungus *Cladosporium fulvum* is not sensitive to the chitinase and β -1,3-glucanase defense proteins of its host, tomato. *Physiol. Mol. Plant Pathol.* 46:45-59.
- Joosten, M. H. A. J., Vogelsang, R., Cozijnsen, T. J., Verberne, M. C., and de Wit, P. J. G. M. 1997. The biotrophic fungus *Cladosporium fulvum* circumvents *Cf-4*-mediated resistance by producing unstable Avr4 elicitors. *Plant Cell* 9:367-379.
- Joris, B., Englebert, S., Chu, C. P., Kariyama, R., Daneomooore, L., Shockman, G. D., and Ghuyssen, J. M. 1992. Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 91:257-264.
- Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103:11086-11091.
- Kirsch, T., Paris, N., Butler, J. M., Beevers, L., and Rogers, J. C. 1994. Purification and initial characterization of a potential plant vacuolar targeting receptor. *Proc. Natl. Acad. Sci. U.S.A.* 91:3403-3407.
- Kruijt, M., de Kock, M. J. D., and de Wit, P. J. G. M. 2005. Receptor-like proteins involved in plant disease resistance. *Mol. Plant Pathol.* 6:85-97.
- Laugé, R., Joosten, M. H. A. J., Haanstra, J. P., Goodwin, P. H., Lindhout, P., and de Wit, P. J. G. M. 1998. Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen. *Proc. Natl. Acad. Sci. U.S.A.* 95:9014-9018.
- Laugé, R., Goodwin, P. H., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2000. Specific HR-associated recognition of secreted proteins from *Cladosporium fulvum* occurs in both host and non-host plants. *Plant J.* 23:735-745.
- Lazarovits, G., and Higgins, V. J. 1976. Histological comparison of *Cladosporium fulvum* race 1 on immune, resistant, and susceptible tomato varieties. *Can. J. Bot.* 54:224-234.
- Li, L., and Steffens, J. C. 2002. Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta* 215:239-247.
- Limpens, E., Franken, C., Smit, P., Willemse J., Bisseling, T., and Geurts, R. 2003. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302:630-633.
- Luderer, R., Rivas, S., Nurnberger, T., Mattei, B., van den Hooven, H. W., van der Hoorn, R. A. L., Romeis, T., Wehrfritz, J. M., Blume, B., Nennstiel, D., Zuidema, D., Vervoort, J., de Lorenzo, G., Jones, J. D. G., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2001. No evidence for binding between resistance gene product Cf-9 of tomato and avirulence gene product AVR9 of *Cladosporium fulvum*. *Mol. Plant-Microbe Interact.* 14:867-876.
- Luderer, R., Takken, F. L. W., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2002. *Cladosporium fulvum* overcomes *Cf-2*-mediated resistance by producing truncated AVR2 elicitor proteins. *Mol. Microbiol.* 45:875-884.
- Mackey, D., Belkadir, Y., Alonso, J. M., Ecker, J. R., and Dangl, J. L. 2003. *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112:379-389.
- Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczygowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and Stougaard, J. 2003. A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425:637-640.
- Mauch, F., Mauchmani, B., and Boller, T. 1988. Antifungal hydrolases in pea tissue .2. inhibition of fungal growth by combinations of chitinase and beta-1,3-glucanase. *Plant Physiol.* 88:936-942.
- Mayer, A. M. 2006. Polyphenol oxidases in plants and fungi: going places? A review. *Phytochemistry* 67:2318-2331.
- Oort, A. J. P. 1944. Onderzoekingen over stuifbrand. II. Overgevoeligheid voor stuifbrand (*Ustilago tritici*). *Tijdschr. Plantenziekten* 50:73-106.
- Perfect, S. E., O'Connell, R. J., Green, E. F., Doering-Saad, C., and Green, J. R. 1998. Expression cloning of a fungal proline-rich glycoprotein specific to the biotrophic interface formed in the *Colletotrichum*-bean interaction. *Plant J.* 15:273-279.
- Ponting, C. P., Aravind, L., Schultz, J., Bork, P., and Koonin, E. V. 1999. Eukaryotic signalling domain homologues in archaea and bacteria. Ancient ancestry and horizontal gene transfer. *J. Mol. Biol.* 289:729-745.
- Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Gronlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425:585-592.
- Raj, S. N., Sarosh B. R., and H. S. Shetty. 2006. Induction and accumulation of polyphenol oxidase activities as implicated in development of resistance against pearl millet downy mildew disease. *Funct. Plant Biol.* 33:563-571.
- Ramonell, K., Berrocal-Lobo, M., Koh, S., Wan, J. R., Edwards, H., Stacey, G., and Somerville, S. 2005. Loss-of-function mutations in chitin responsive genes show increased susceptibility to the powdery mildew pathogen *Erysiphe cichoracearum*. *Plant Physiol.* 138:1027-1036.
- Ramonell, K. M., Zhang, B., Ewing, R. M., Chen, Y., Xu, D., Stacey, G., and Somerville, S. 2002. Microarray analysis of chitin elicitation in *Arabidopsis thaliana*. *Mol. Plant. Pathol.* 3:301-311.
- Ridout, C. J., Skamnioti, P., Porritt, O., Sacristan, S., Jones, J. D. G., and Brown, J. K. M. 2006. Multiple avirulence paralogs in cereal powdery mildew fungi may contribute to parasite fitness and defeat of plant resistance. *Plant Cell* 18:2402-2414.
- Rooney, H. C., van 't Klooster, J. W., van der Hoorn, R. A. L., Joosten, M. H. A. J., Jones, J. D. G., and de Wit, P. J. G. M. 2005. *Cladosporium* Avr2 inhibits tomato Rcr3 protease required for *Cf-2*-dependent disease resistance. *Science* 308:1783-1786.
- Schlumbaum, A., Mauch, F., Vogeli, U., and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. *Nature* 324:365-367.

- Sela-Buurlage, M. B., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S., Van den Elzen, P. J. M., and Cornelissen, B. J. C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and beta-1,3-glucanases exhibit antifungal activity. *Plant Physiol.* 101:857-863.
- Shibuya, N., and Minami, E. 2001. Oligosaccharide signaling for defense responses in plants. *Physiol. Mol. Plant Pathol.* 59:223-233.
- Shimada, T., Fuji, K., Tamura, K., Kondo, M., Nishimura, M., and Hara-Nishimura, I. 2003. Vacuolar sorting receptor for seed storage proteins in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 100:16095-16100.
- Takken, F. L. W., Schipper, D., Nijkamp, H. J. J., and Hille, J. 1998. Identification and Ds-tagged isolation of a new gene at the *Cf-4* locus of tomato involved in disease resistance to *Cladosporium fulvum* race 5. *Plant J.* 14:401-411.
- Terras, F. R. G., Schoofs, H. M. E., De Bolle, M. F. C., Van Leuven, F., Rees, S. B., Vanderleyden, J., Cammue, B. P. A., and Broekaert, W. F. 1992. Analysis of two novel classes of antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.* 267:15301-15309.
- Terras, F. R. G., Torrekens, S., Van Leuven, F., Osborn, R. W., Vanderleyden, J., Cammue, B. P. A., and Broekaert, W. F. 1993. A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae-species. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 316:233-240.
- Thipyapong, P., Hunt, M. D., and Steffens, J.C. 2004. Antisense down-regulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta* 220:105-107.
- Thomas, C. M., Jones, D. A., Parniske, M., Harrison, K., Balint-Kurti, P. J., Hatzixanthis, K., and Jones, J. D. G. 1997. Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognition specificity in Cf-4 and Cf-9. *Plant Cell* 9:2209-2224.
- Thomma, B. P. H. J. 2003. *Alternaria* spp.: from cosmopolitan saprophyte to specific parasite. *Mol. Plant Pathol.* 4:225-236.
- Thomma, B. P. H. J., Eggermont, K., Penninckx, I. A. M. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A., and Broekaert, W. F. 1998. Separate jasmonate-dependent and salicylate-dependent defense response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 95:15107-15111.
- Thomma, B. P. H. J., Eggermont, K., Broekaert, W. F., and Cammue, B. P. A. 2000. Disease development of several fungi on *Arabidopsis* can be reduced by treatment with methyl jasmonate. *Plant Physiol. Biochem.* 38:421-427.
- Thomma, B. P. H. J., Penninckx, I. A. M. A., Broekaert, W. F., and Cammue, B. P. A. 2001. The complexity of disease signaling in *Arabidopsis*. *Curr. Opin. Immunol.* 13:63-68.
- Thomma, B. P. H. J., van Esse, H. P., Crous, P. W., and de Wit, P. J. G. M. 2005. *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Mol. Plant Pathol.* 6:379-393.
- Thomma, B. P. H. J., Bolton, M. D., Clergeot, P. H., and de Wit, P. J. G. M. 2006. Nitrogen controls in planta expression of *Cladosporium fulvum* Avr9 but no other effector genes. *Mol. Plant Pathol.* 7:125-130.
- Tian, M., Huitema, E., Da Cunha, L., Torto-Alalibo, T., and Kamoun, S. 2004. A Kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. *J. Biol. Chem.* 279:26370-26377.
- Tian, M., Benedetti, B., and Kamoun, S. 2005. A second Kazal-like protease inhibitor from *Phytophthora infestans* inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato. *Plant Physiol.* 138:1785-1793.
- Tian, M., Win, J., Song, J., van der Hoorn, R. A. L., van der Knaap, E., and Kamoun, S. 2007. A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. *Plant Physiol.* 143:364-377.
- Ueda, H., Yamaguchi, Y., and Sano, H. 2006. Direct interaction between the Tobacco mosaic virus helicase domain and the ATP-bound resistance protein, N factor during the hypersensitive response in tobacco plants. *Plant Mol. Biol.* 61:31-45.
- van den Ackerveken, G. F. J. M., van Kan, J. A. L., Joosten, M. H. A. J., Muissers, J. M., Verbakel, H. M., and de Wit, P. J. G. M. 1993. Characterization of two putative pathogenicity genes of the fungal tomato pathogen *Cladosporium fulvum*. *Mol. Plant-Microbe Interact.* 6:210-215.
- van den Ackerveken, G. F. J. M., Dunn, R. M., Cozijnsen, A. J., Vossen, J. P. M. J., van den Broek, H. W. J., and de Wit, P. J. G. M. 1994. Nitrogen limitation induces expression of the avirulence gene *Avr9* in the tomato pathogen *Cladosporium fulvum*. *Mol. Gen. Genet.* 243:277-285.
- van den Burg, H. A., Westerink, N., Francoijs, K. J., Roth, R., Woestenenk, E., Boeren, S., de Wit, P. J. G. M., Joosten, M. H. A. J., and Vervoort, J. 2003. Natural disulfide bond-disrupted mutants of AVR4 of the tomato pathogen *Cladosporium fulvum* are sensitive to proteolysis, circumvent Cf-4-mediated resistance, but retain their chitin binding ability. *J. Biol. Chem.* 278:27340-27346.
- van den Burg, H. A., Spronk, C. A., Boeren, S., Kennedy, M. A., Vissers, J. P., Vuister, G. W., de Wit, P. J. G. M., and Vervoort, J. 2004. Binding of the AVR4 elicitor of *Cladosporium fulvum* to chitotriose units is facilitated by positive allosteric protein-protein interactions: the chitin-binding site of AVR4 represents a novel binding site on the folding scaffold shared between the invertebrate and the plant chitin-binding domain. *J. Biol. Chem.* 279:16786-16796.
- van den Burg, H. A., Harrison, S. J., Joosten, M. H. A. J., Vervoort, J., and de Wit, P. J. G. M. 2006. *Cladosporium fulvum* Avr4 protects fungal cell walls against hydrolysis by plant chitinases accumulating during infection. *Mol. Plant-Microbe Interact.* 19:1420-1430.
- van der Hoorn, R. A. L., Laurent, F., Roth, R., and de Wit, P. J. G. M. 2000. Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis. *Mol. Plant-Microbe Interact.* 13:439-446.
- van Esse, H. P., Thomma, B. P. H. J., van't Klooster, J. W., and de Wit, P. J. G. M. 2006. Affinity-tags are removed from *Cladosporium fulvum* effector proteins expressed in the tomato leaf apoplast. *J. Exp. Bot.* 57:599-608.
- van Kan, J. A. L., van den Ackerveken, G. F., and de Wit, P. J. G. M. 1991. Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. *Mol. Plant-Microbe Interact.* 4:52-59.
- Weng, L., Dai, H., Zhan, Y., He, Y., Stepaniants, S. B., and Bassett, D. E. 2006. Rosetta error model for gene expression analysis. *Bioinformatics* 22:1111-1121.
- Westerink, N., Roth, R., van den Burg, H. A., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2002. The AVR4 elicitor protein of *Cladosporium fulvum* binds to fungal components with high affinity. *Mol. Plant-Microbe Interact.* 15:1219-1227.
- Westerink, N., Brandwagt, B. F., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2004. *Cladosporium fulvum* circumvents the second functional resistance gene homologue at the *Cf-4* locus (*Hcr9-4E*) by secretion of a stable *avr4E* isoform. *Mol. Microbiol.* 54:533-545.
- Wubben, J. P., Eijkelboom, C. A., and de Wit, P. J. G. M. 1993. Accumulation of pathogenesis-related proteins in the epidermis of tomato leaves infected by *Cladosporium fulvum*. *Neth. J. Plant Pathol.* 99:231-239.