The *Cladosporium fulvum* Avr2 protein behaves both as a virulence and an avirulence factor

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CHAPTER 1

Gene-for-gene models:
direct and indirect interactions between pathogen effectors and cognate host resistance proteins

Plants are not able to move or escape and have to confront environmental challenges like nutrient and water deprivation, low and high temperatures, different abiotic stresses and biotic stresses imposed by pathogens like viruses, bacteria, fungi, nematodes and insects, that all compete for plant nutrient sources. The outcome of a plant-pathogen interaction can vary from mild symptoms that are hardly harmful to the host to complete destruction of the host plant.

During evolution pathogens acquired mechanisms of evasion or suppression of basal host defense on the one hand and plants have evolved various mechanisms to counter-attack infections by pathogens on the other hand.
Specific recognition of a pathogen by its host and activation of downstream defense signaling are complex and both organisms have to come up with sophisticated strategies to survive their encounters.

These encounters have in principle two possible outcomes: (i) a pathogen successfully infects its host plant, which is also referred to as a compatible interaction (the pathogen is virulent and the host plant is susceptible), or (ii) the pathogen cannot successfully infect its host plant which stays healthy, also referred to as an incompatible interaction (the pathogen is avirulent and the host plant is resistant).
Nearly 70 years ago, Harold Flor (1942) studied the genetics of the interaction between the flax rust fungus *Melampsora lini* and flax, *Linum usitatissimum*. Based on these studies he postulated the so-called gene-for-gene hypothesis (Flor, 1942) which states that for each dominant resistance (*R*) gene in the host there is a cognate dominant avirulence (*Avr*) gene in the pathogen. Co-occurrence and expression of both genes leads to an incompatible interaction that is often associated with a hypersensitive response (HR), a type of programmed cell death in the region surrounding the primary infection site.
In the introduction of my thesis I will focus mainly on physiological, genetic, molecular and biochemical aspects of interactions between host plants and their pathogenic bacteria, fungi and oomycetes.

1. *Avr* gene products or elicitors

In gene-for-gene interactions, pathogen avirulence (*Avr*) gene products elicit a resistance reaction in plants that carry a cognate resistance (*R*) gene, which is usually associated with an HR. It is assumed that the Avr protein elicits the cognate plant R protein which is followed by R-mediated downstream defense signaling pathways, culminating in HR and phytoalexin accumulation. For this reason Avr proteins were previously called elicitors and initially they were only allowed to be called elicitors when they induced the accumulation of phytoalexins to levels that inhibited fungal growth in the host plant at the site of infection (Keen, 1975).

Thus, according to this definition, elicitors were initially identified by their ability to trigger *R* gene-mediated HR and accumulation of phytoalexins. Since Flor coined the gene-for-gene hypothesis many biologists, plant scientists and pathologists have studied different pathosystems to find experimental evidence for the gene-for-gene hypothesis by molecular and biochemical approaches. However, it took over forty years before Flor's gene-for-gene hypothesis could be proven experimentally (Staskawicz et al., 1984).

The cloning of the first bacterial *Avr* gene, *avrB*, was achieved by the construction of a cosmid-based genomic library of an avirulent strain of *Pseudomonas syringae* pv. *glycinea* (Psg)*,* that was mobilized into a virulent strain of this bacterium and the transformants obtained were assayed for transition from virulent to avirulent on soybean (*Glycine max)* cultivar Harosoy carrying the cognate *R* gene (Staskawicz et al., 1984).

A few years earlier research on the *Cladosporium fulvum*-tomato interaction demonstrated the presence of *Cf* gene-specific elicitors of HR in intercellular fluids recovered from the apoplast of tomato leaves inoculated with a virulent race of this fungus containing the cognate *Avr* gene. (De Wit and Spikman, 1982) It took nearly fifty years after Flor's postulation of the gene-for-gene hypothesis before the first fungal *Avr* gene was cloned (*Avr9* of *C. fulvum*) by a reverse genetics approach (van Kan et al., 1991), whereas it took more than 60 years before the first oomycete *Avr* gene was cloned (*Avr1b* gene of *Phytophthora sojae*) by a map-based cloning approach (Shan et. al., 2004).

Presently, numerous bacterial and fungal and oomycete *Avr* genes have been cloned (Block et. al., 2008; Stergiopoulos and de Wit, 2009; Kamoun, 2007).

In the following section, we will discuss a selection of cloned bacterial and fungal *Avr* genes and their cognate plant *R* genes in more detail.

1.1. Bacterial *Avr* gene-*R* gene interactions

The discovery that many *Pseudomonas syringae* species could elicit an HR in non-host plants was important for further development of experimental bacterial-plant pathosystems (Klement, 1963) The intensive research on *P. syringae*-plant interactions has led to the identification of a number of *Avr* and *hrp (*hypersensitive response and pathogenicity) genes. *Hrp* genes are required for the activation of HR in non-host and resistant plants and for induction of pathogenicity in susceptible host plants (Lindgren et al., 1988). Mutation analysis of *P. syringae hrp* genes showed that they all encode proteins that function as building blocks of the machinery for the bacterial type three secretion system (TTSS) which is required for delivery of Avr proteins by injection into host plants (Figure 1). Significantly, *P. syringae Avr* genes are co-regulated with *hrp* genes and are dependent on the *hrp* regulatory system (Salmeron et al., 1993; Lorang and Keen, 1995; Dangl et al., 1994).

All *hrp* mutants lack a functional TTSS, are not virulent on susceptible plants and can no longer induce a typical HR on plants carrying the cognate *R* gene. This suggests that bacteria need a functional TTSS to cause disease symptoms on susceptible plants, and furthermore that plants contain a specific *R*-gene encoded perception system to recognize and to subsequently inhibit bacterial multiplication until a critical threshold has been reached where host damaging disease symptoms can no longer develop (Lindgren, 1997).

1.2. The *Ralstonia solanacearum (PopP2)-Arabidopsis thaliana* (*RRS1-R/RD19*) interaction

The soil-born vascular pathogen *Ralstonia solanacearum*, causal agent of bacterial wilt, has been shown to infect a range of *Arabidopsis thaliana* accessions, which has been exploited to identify the *RRS1-R* (Resistance to *Ralstonia solanacearum1-R*) gene (Deslandes et. al., 2002). *RRS1-R* encodes a TIR-NB-LRR (Toll and Interleukin Receptor- Nucleotide Binding site-Leucine Rich Repeat) protein with a C-terminal WRKY motif which is characteristic for a particular class of plant transcriptional regulators (Eulgem and Somssich, 2007). During infection *R. solanacearum* delivers a number of effector proteins by its TTSS, which have been designated Pops (*Pseudomonas* outer proteins) and belong to the YopJ/AvrRxv family, which contains members that have been proposed to act as cysteine proteases (Orth, 2002). One member, PopP2, behaves as an Avr factor in *A. thaliana* accessions containing the *RRS1-R* gene, and by using the yeast split-ubiquitin two-hybrid system it was shown that PopP2 specifically interacts with the RRS1-R protein. PopP2 contains a bipartite nuclear localization signal and it was demonstrated that localization of RRS1-R is PopP2-dependent (Deslandes et. al., 2003). In a later study it was shown that a third interacting protein of host origin is important for the nuclear localization of PopP2 and RRS1-R. This is the vacuole-localized cysteine protease RD19 (Responsive to Dehydration 19) which interacts with both PopP2 and RRS1-R. RD19 is also required

for RRS1-R-mediated resistance (Bernoux et al., 2008). During bacterial infection and co-expression of PopP2, the expression of *RD19* is induced in the vascular tissue. RD19 specifically re-localizes to the plant nucleus, where the two proteins physically interact. Although RRS1-R and RD19 interact *in vitro* their interaction in the nucleus in the presence of PopP2 could not be shown yet. It is hypothesized that RD19 forms a nuclear complex with PopP2 which is subsequently required for RRS1-R-mediated resistance (Bernoux et al., 2008). Interestingly, RRS1-R is identical to SHL1 (Sensitivity to Low Humidity 1) except for the amino acid insertion in the WRKY domain leading to a loss of DNA-binding activity. It is assumed that in absence of infection RRS1-R is bound to promoter(s) of defense genes to suppress their expression, and that in *R. solanacearum*-infected plants the interaction of PopP2 with RRS1-R displaces RRS1-R from the promoter(s) after which defense gene expression occurs (Shen and Schulze-Lefert, 2007).

1.3. *Pseudomonas syringae* pv. *tomato (avrRpt2)*-tomato (*RPS2*) interaction

The Pst *(Pseudomonas syringae* pv. *tomato)-A. thaliana* pathosystem has served as an excellent model to study the genetic, molecular and biochemical basis of the gene-for-gene interaction and to find evidence for direct interaction between a bacterial Avr factor and the cognate plant R protein. The avirulence gene *avrRpt2* of Pst was identified through screening of a genomic cosmid library of a Pst strain (avirulent on ecotype Sf-2) when transferred into Pst DC3000 (virulent on *A. thaliana* ecotype Col-0) which became avirulent when the cosmid harbored the *avrRpt2* gene. Further proof that the avirulence gene had been cloned came from transferring the *avrRpt2* gene from Pst into virulent strains of the soybean pathogen Psg which led in all cases to an avirulent phenotype on soybean cultivar Harosoy. This indicates that similar resistance mechanisms occur and are functional in both *A. thaliana* and soybean (Whalen et al., 1991).
The *avrRpt2* gene encodes a 28.2 kDa protein and its expression depends on the *hrpRS* regulatory locus (Innes et al., 1993). Genetic analyses of *A. thaliana* mutants of which the parental ecotype generated an HR upon inoculation with a Pst strain harboring the *avrRpt2* gene showed that some mutants had become fully susceptible to this strain. The mutants contained alterations in a single locus, named *RPS2* (*Resistance to Pseudomonas syringae 2*). Heterozygous *RPS2/rps2* plants displayed a phenotype intermediate between that of *RPS2/RPS2* and *rps2/rps2* (Yu et al., 1993). Remarkably, *rps2/rps2* mutants retained resistance to Pst strains expressing avirulence gene *avrB* from Psg or the *avrRpm1* gene from *P. syringae* pv. *maculicola* (Kunkel et al., 1993). The *RPS2* gene was isolated by map-based cloning and shown to encode a 105 kDa CC-NB-LRR (Coiled Coil-Nucleotide Binding site-Leucine Rich Repeat) protein localized in the cytoplasm (Mindrinos et al., 1994; Bent et al., 1994). Transient expression of *avrRpt2* elicits an HR in plants containing *RPS2,* demonstrating that no additional bacterial factors other than the *avrRpt2* gene

product are required for induction of an HR. Similar observations were made for AvrRpm1-triggered RPM1-mediated resistance (Leister et al., 1996). The AvrRpt2 protein is a cysteine protease and is N-terminally processed after secretion. This N-terminus is not required for recognition by RPS2, but is essential for its trafficking through the TTSS (Mudgett et al., 1999). Mutational analysis of the catalytic residues of Avr2Rpt2 resulted in (i) elimination of the *in planta* processing, (ii) blocking of processing of RIN4 (discussed later), (iii) reduction of virulence and, (iv) inactivation of the RPS2-mediated resistance response (Axtell et al., 2003a). Downstream of Avr2Rpt2-triggered RPS2-mediated activation of defense many additional components are essential for efficient defense signaling such as RAR1, SGT1 and HSP90. The production of *A. thaliana* cytosolic AtHSP90.1 is rapidly induced upon challenge by Pst DC3000 carrying *avrRpt2* and is required for complete RPS2-mediated resistance. RAR1 and SGT1 function as chaperones of HSP90 which is essential for functioning of the RPS2 protein (Takahashi et al., 2003). Transgenic plants expressing *avrRpt2* showed enhanced susceptibility to Pst DC3000 strains lacking the TTSS, indicating that AvrRpt2 is a virulence factor important for suppression of basal defense responses.
In the cytosol of the host AvrRpt2 is folded into an active cysteine protease by the host cyclophilin ROC1 (Rotamase Cyp1) (Coaker et al., 2005) after which it can target RIN4 and RIN4-related proteins.

1.3.1. A role for RIN4 (RPM1-interacting protein 4)

In *A. thaliana* the interaction between AvrRpt2 and RPS2 antagonizes the interaction between AvrRpm1 and RPM1. This antagonism suggests competition for one or more common components in a signal transduction pathway leading to *R* gene-mediated resistance (Ritter et al., 1996). A study with mutant versions of *A. thaliana* RPS2 revealed that the protein interacts with a 75-kD protein (Tao et al., 2000). The interacting protein was isolated by co-immunoprecipitation with cell extracts containing AvrB, AvrRpm1 or RPM1 and named RIN4 (RPM1 interacting protein 4). Reduction of *Rin4* expression showed inhibition of the RPM1-mediated HR and a decrease of virulence of Pst but, interestingly, also of the oomycete *Peronospora parasitica* (recently renamed *Hyaloperonospora arabidopsidis,* Hpa). This indicates that RIN4 (i) positively regulates RPM1-mediated resistance and (ii) negatively regulates basal defense. The AvrRpm1 and AvrB effectors both induce RIN4 phosphorylation which leads to negative regulation of basal defense and increased virulence of the pathogen in the absence of RPM1. In RPM1-mediated resistance, RPM1 guards RIN4 and after detection of conformational changes of RIN4 an HR is mounted that is effective towards pathogens expressing AvrB or AvrRpm1. This phenomenon is also known as the “guard” model (Mackey et al., 2002), a term that was initially coined by van der Biezen and Jones (1998). AvrRpt2 is unrelated to AvrB and AvrRpm1 but also targets, processes and eliminates RIN4 independent of

the presence of RPS2. In plants containing the *Rps2* gene, RIN4 physically interacts with RPS2 and elimination of RIN4 activates the RPS2-mediated defense signaling pathway (Mackey et al., 2003). RIN4 elimination by AvrRpt2 also occurs in *rps2* and *ndr1* mutant plants proving that the elimination is independent of an RPS2-mediated signaling pathway. RPS2-mediated resistance is based on perception of RIN4 elimination (Axtell et al., 2003b). The activation of RPS2 appeared to be NDR1-independent (Belkhadir et al., 2004) (to be discussed later). AvrRpt2 virulence activity likely relies on proteolysis of RIN4, in which the amino acid sequence surrounding the cleavage site is highly conserved in two locations of the RIN4 protein, designated RCS1 and RCS2 (RIN4 cleavage site 1 and 2, respectively), and one in the AvrRpt2 protein designated ACS (AvrRpt2 cleavage site). This demonstrates that AvrRpt2 not only cleaves RIN4-like proteins, but has also autocatalytic activity (Lim et al., 2004). AvrRpt2 mutants deficient in N-terminal autoprocessing were still able to eliminate RIN4, whereas the proteolytically deficient *avrRpt2* mutant (carrying the Cys122>Ala amino acid substitution) was also deficient in N-terminal autoprocessing and RIN4 elimination. The growth rate of Pst expressing AvrRpt2 on *A. thaliana rin4/rps2* was significantly higher as compared to the catalytically inactive AvrRpt2 mutants suggesting that, in addition to RIN4, AvrRpt2 targets other host components. Several of these target proteins were eliminated in an AvrRpt2-dependent manner in a transient *in planta* expression system (Chisholm et al., 2005). It was demonstrated that *in planta* the cleavage of RCS2 is critical and leads to AvrRpt2-mediated elimination of a 6.4 kDa C-terminal fragment of RIN4 which is important for its localization to the host plasma membrane. This C-terminal fragment harbors three consecutive cysteine amino acids of which at least one is required for linking RIN4 to the plasma membrane, which occurs likely through palmitoylation and/or prenylation. As a consequence of this cleavage the remainder of RIN4 is released from the membrane and prevents RPM1 activation by AvrRpm1 or AvrB. The RCS2 cleavage site is surrounded by a 30 amino acid sequence important for binding to AvrB which has a plant-specific signature and is found in a small *A. thaliana* protein family. AvrRpt2 and AvrB target the same domain of RIN4 and possibly other RIN4-like proteins (Kim et al., 2005). Also specific amino acid residues within the C-terminal part of RIN4 are required for the association with RPS2 (Day et al., 2005). In susceptible host plants RIN4 is the virulence target of three unrelated Pst effector proteins, AvrB, AvrRpm1 and AvrRpt2, and maybe also of fungal and oomycetous effectors. In resistant plants RIN4 seems to represent a point of convergence for the achievement of RPS2- and RPM1-mediated defense signaling and resistance (Mackey et al., 2003; Axtell et al., 2003b). Also in other host plants RIN4-like proteins appear to be important for interaction with pathogens. In lettuce many *RIN4*-like genes exist that appear to be involved in resistance toward the oomycete downy mildew pathogen *Bremia lactucae* (Jeuken et al., 2009).

1.3.2. A role for *NDR*, a gene involved in non race-specific disease resistance

A screening of mutagenized *A. thaliana* plants revealed an *ndr1-1* (non race-specific disease resistance or nonspecific disease resistance 1) mutant that was susceptible to Pst DC3000 harboring *avrRpt2* but resistant to strains expressing *avrB* and *avrRpm1*. Interestingly, this *ndr1-1* mutant appeared also susceptible to the oomycete Hpa, suggesting that *Ndr1* is a common factor required in a defense signaling pathway active towards two unrelated pathogens (Century et al., 1995).The *Ndr1* gene encodes a 219 amino acid protein with two putative transmembrane domains, suggesting that it might be associated with a membrane. Expression analysis of *Ndr1* upon challenge with pathogens showed that NDR1 may function as an integration modulator in recognition of various pathogens (Century et al., 1997). As discussed above, AvrRpt2-RIN4-RPS2 and AvrRpm1-RIN4-RPM1 signaling pathways show differential mechanisms of activation but both share the requirement for NDR1. NDR1 interacts with RIN4 through its cytoplasmic N-terminal part, which is required for AvrRpt2-triggered RPS2-mediated resistance (Day et al., 2006).

2. Eukaryotic pathogen-host interactions

Eukaryotic plant pathogens effectors have been much less intensively studied as compared to bacterial effectors.

The (activities of) secreted Avr proteins of *C. fulvum* are perceived directly or indirectly by extracellular tomato Cf resistance proteins. Other fungal and oomycete effectors exert their activity either in the extracellular matrix or inside the plant host cell. How these effectors are delivered into the host cell and contribute to pathogen virulence is often unknown. A few oomycete- and fungus-host interactions will be discussed here in more detail.

2.1. Oomycete *Avr* gene-plant *R* gene interactions

A distinctive group of very destructive pathogens such as *Phytophthora infestans*, *Hyaloperonospora arabidopsidis* and *Albugo candida* belong to the oomycetes. Oomycetes were considered fungi until a few decades ago, but they are more distantly related to true fungi than fungi to man. Oomycetes grow in a filamentous way similar to fungi, but they are related to lower plants like brown algae, although they lack chlorophyll. Oomycetes are diploid organisms (Agrios 2005)

For many years oomycete genetic research has been considered the dark area of plant-microbe interactions. Effectors of *Phytophthora* species appeared to be modular and can be divided in the extracellular proteins secreted in the host apoplast and proteins that are directed into the cytoplasm of the host where they are recognized by cognate cytoplasmic R proteins. Extracellular elicitins are small, highly conserved proteins secreted by species of *Phytophthora* and *Pythium* that induce necrosis in plants and elicit an HR-like reaction. The elicitin Inf1 can be considered

as a pathogen-associated molecular pattern (PAMP) occurring in most oomycetes (Vleeshouwers et al., 2006).
EPIs (Extracellular Proteinase Inhibitors) have been shown to act as protease inhibitors and can be divided in two families. The family of Kazal-like serine protease inhibitors consists of at least 35 members, and is present in at least five different *Phytophthora* species (Tian et al., 2004). EPI1 and EPI10 inhibit the tomato serine protease P69B (Tian et al., 2005a, 2005b). Another family of proteins, the so-called EPICs (EPI Cystatin-like domains), consists of at least four members of which EPIC1-4 and EPIC2B were shown to interact with, and inhibit, PIP1 (Phytophthora Induced Protein 1) a tomato cysteine protease (Tian et al., 2007). A recent study shows that EPIC1 and EPIC2B can also target the tomato cysteine protease Rcr3, which also interacts with the Avr2 effector protein of *C. fulvum.* These results demonstrate that effectors from phylogenetically unrelated pathogens can target the same host defense proteins (Song et al., 2009). Interestingly, unlike Avr2, EPIC1 and EPIC2B do not trigger an HR or other defense responses on Cf-2/Rcr3 tomato.
A number of intracellular cytoplasmic oomycete effectors has been divided in two families (i) the N- terminal RXLR containing effectors, and (ii) the CNRs (Crinklers). Both types are translocated to the cytoplasm of host cells (Kamoun, 2006, 2007). So far, only oomycete effectors belonging to the RXLR family are recognized by NB-LRR resistance proteins. The effector genes are often up-regulated during the biotrophic phase of the infection and are involved in cell death suppression. By using an *Agrobacterium tumefaciens* transient assay system performed in *Nicotiana benthamiana*, it has been shown that the RXLR effector Avr3aKI (the K80 and I103 variant) interacts with the cognate *R3a* gene product and triggers cell death, but Avr3aKI also suppresses cell death induced by Inf1 (Bos et al., 2006). The Avr3aEM mutant protein activates an R3a-mediated HR but does not suppress Inf1-induced cell death (Bos et al., 2009). Thus Avr3a can be considered a suppressor of Inf1-triggered immunity. Over-expression of the RXLR effector *Avr1b* in *P. sojae* results in increased virulence on susceptible host plants and suppression of cell death induced by the pro-apoptotic BAX protein in both yeast and plants (Dou et al., 2008). Recombinant green fluorescent protein containing the RXLR domain directly enters soybean root cells suggesting that uptake of RXLR effectors requires host import machinery and does not require a pathogen-provided translocation machinery such as the TTSS reported for bacteria (Dou et al., 2008). Whole genome sequencing of several oomycete genomes has revealed the existence of hundreds of effector genes in these genomes, suggesting that oomycetes need to perturb many host targets in order to successfully infect their host plants. Dissecting the individual virulence functions of so many effectors remains a main challenge for future research.

3. Fungal *Avr* gene-plant *R* gene interactions

3.1 The *Rhynchosporium secalis*-barley pathosystem

Studies on the *Rhynchosporium secalis*-barley pathosystem revealed that the fungus secretes three low-molecular-weight peptides Nip1, Nip2 and Nip3 (Necrosis-inducing peptides 1, 2 and 3). In bioassays, these peptides elicit non-specific necrosis in primary leaves of both resistant and susceptible barley cultivars (Wevelsiep et al., 1991). Nip1 (renamed AvrRrs1) elicits specific (non-HR) defense responses in barley cultivars harboring the *Rrs1* resistance gene (Hahn et al., 1993). Mature Nip1 shows all common features of a fungal elicitor and contains 10 cysteines which are all involved in intramolecular disulfide bonds (Rohe et al., 1995; Van't Slot et al., 2003). Virulent strains of *R. secalis* on *Rrs1* barley plants either lack the *Nip1* gene or the sequence of the *Nip1* gene contains point mutations that result in single amino acid substitutions (Rohe et al., 1995). Three Nip1 isoforms have been described that contain single amino acid substitutions which are correlated with gain of virulence on Rrs1 plants. To date, 14 Nip1 isoforms have been identified and it has been reported that the deletion frequency of *Nip1* was higher in comparison to *Nip2* and *Nip3*. Apparently, loss of this gene does not affect the fitness of the fungus. Nip1 interacts with a single high affinity plasma membrane receptor that is involved in both virulence and Rsr1-mediated defense, but the gene for this receptor and the *Rsr1* gene have not been characterized yet (Van't Slot et al., 2007).

3.2 The *Fusarium oxysporum* f. sp. *lycopersici* - tomato pathosystem

The vascular pathogenic fungus *F. oxysporum* f. sp. *lycopersici* secretes several proteins in the xylem of the host during infection. To date four so-called Six effectors (Secreted in xylem) have been cloned. Six1 (renamed Avr3) and Six3 (renamed Avr2) are required for full virulence on tomato (Rep et al., 2004, 2005), but also trigger a defense response in the presence of the cognate *I-2* (Houterman et al., 2009) and *I-3* resistance genes, respectively (Huang and Lindhout, 1997; Rep et al., 2004). It is notable that all three *Six* genes are located on a small chromosome only found in the *F. oxysporum* f. sp. *lycopersici* lineage, and not in other formae speciales or non-pathogenic *Fusarium* species, suggesting that this chromosome is required for virulence on tomato (Van der Does et al., 2008; Ma et al., 2010). This chromosome is probably obtained only once during evolution of the pathogen carrying all the essential effector genes required for infection of tomato. This has probably been achieved by means of horizontal gene transfer in one strain that has subsequently spread to other clonal *F. oxysporum* f. sp. *lycopersici* strains (van der Does et al., 2008; Ma et al., 2010). Recently it has been shown that non-pathogenic *Fusarium* strains can obtain this chromosome from pathogenic strains by co-cultivation on artificial media (Ma et al., 2010). In contrast to the previous Six proteins, Six4 (renamed Avr1) is not required for full virulence of *F. oxysporum* f. sp.

lycopersici strains on susceptible tomato plants and provides avirulence to the strains on tomato lines carrying the *I* or *I-1* resistance gene. Transformation of *Avr1* in *F. oxysporum* f. sp. *lycopersici* strains avirulent on *I-2* and/or *I-3* tomato lines show a gain of virulence on these lines. This demonstrates that Avr1 functions as a suppressor of both *I-2*- and *I-3*-mediated resistance. Interestingly, to date all *F. oxysporum* f. sp. *lycopersici* strains analyzed contain *Avr3*, whereas *Avr1* is only present in strains that are virulent on *I-2* and/or *I-3* lines. Even if *Avr1* is not required for full virulence it might have been acquired to compensate for the fitness penalty due the loss of *Avr3* and probably also *Avr2* in overcoming *I-2*- and *I-3*-mediated resistance. Remarkably, the Avr2 protein is secreted in the xylem of tomato during fungal colonization but is recognized in the cell by I-2 protein, suggesting that it is taken up by the host. Single amino acid substitutions found in natural strains result in gain of virulence on *I-2* genotypes (Houterman et al., 2009).

3.3 The *Magnaporthe oryzae*-rice pathosystem

The simplest model for gene-for-gene interactions is the receptor-ligand model, representing direct interaction between the plant R protein and its cognate pathogen Avr protein. An interaction complying with this model is that between *Magnaporthe grisea* Avr-Pi-ta and the rice *Pi-ta R* gene product, a cytoplasmic CC-NB-LRR protein. A number of *M. grisea Avr* genes has been cloned and characterized (Orbach et al., 2000; Valent et al., 1991). I will focus only on the *Avr–Pi-ta* gene, currently renamed *Avr-Pi-ta1.*

Avr-Pi-ta codes for a 233 amino acid protein homologous to fungal zinc dependent metalloproteases with a functional N-terminal secretion signal and pro-protein sequences as demonstrated by transient expression of *Avr-Pi-ta*$_{176}$ which lacks the secretion signal representing the mature Avr-Pi-ta. By yeast two-hybrid assays and in an *in vitro* binding assay (far western) it was proven Avr-Pi-ta$_{176}$ specifically binds to the LRR domain of Pi-ta. Susceptible plants carry an amino acid substitution (Ala918>Ser) in the LRR domain of Pi-ta. Single amino acid substitutions in the Pi-ta LRR domain or in the AVR-Pi-ta$_{176}$ protease motif that result in loss of resistance also abolish physical interaction between the two proteins, both in yeast two-hybrid assays and in an *in vitro* binding assay (Jia et al., 2000). Pi-ta shows low constitutive expression in both resistant and susceptible rice. Rice varieties reported to have the linked *Pi-ta*$_2$ gene contain Pi-ta plus at least one other *R* gene, potentially explaining the broadened resistance spectrum of *Pi-ta*$_2$ relative to *Pi-ta* (Bryan et al., 2000). Various *Avr-Pi-ta* mutations allow the pathogen to circumvent *Pi-ta*-mediated resistance. Interestingly, genetic mapping showed that *Avr-Pi-ta* in *M. grisea* is located entirely within the most distal part of chromosome 3 and spontaneous loss of *Avr-Pi-ta* appears to be related to its telomeric location (Orbach et al., 2000).

Also an insertion of the Pot3 transposon in the promoter of Avr-Pi-ta caused gain of virulence on *Pi-ta*-containing plants (Kang et al., 2001).

Analysis of *M. grisea* isolates from diverse hosts demonstrated that *Avr-Pi-ta1* belongs to a gene family with *Avr-Pi-ta2* and *Avr-Pi-ta3* as members. Avr-Pi-ta2 but not Avr-Pi-ta3 elicits Pi-ta-mediated defense responses. *Avr-Pi-ta1* and *Avr-Pi-ta2* are found in both *M. oryzae* and *M. grisea* but *Avr-Pi-ta3* is only present in *M. oryzae* isolates (Chang et al., 2008). Recently, it was reported that alternative splicing of *Pi-ta* may increase protein diversity.
Gene expression analysis in a resistant host plant containing *Pi-ta* revealed that transcript variants encoding the C-terminal thioredoxin (TRX) domain showed much higher expression levels in comparison to the other full length or truncated transcripts. This suggests that some variants may have a significant function in *R* gene regulation (Costanzo et al., 2009). Genetic analysis of mutated susceptible host plants expressing *Pi-ta* resulted in the identification of the Ptr(t) (Pi-ta resistance) locus, which appeared specific to Pi-ta-mediated signal recognition. This result suggests that a third component involved in the Avr-Pi-ta-Pi-ta interaction is required. To date, Ptr(t) has not yet been characterized (Jia et al., 2008).

3.4 The *Melampsora lini*-flax pathosystem

In contrast to other well-studied pathosystems, information on rust pathogenicity mechanisms is limited due to the fact that rust fungi are obligate biotrophs and difficult to culture or transform. During infection, feeding structures known as haustoria are produced to obtain nutrients from the host. At the interface between the haustorium and the host plasma membrane a dynamic interaction between pathogen and host occurs, involving extensive trafficking of nutrients and signaling molecules. In flax rust-resistant plants HR-mediated resistance is associated with the formation of haustoria (Dodds et al., 2007). As mentioned earlier, the genetic studies by Harold Flor on the interaction between the flax rust fungus *M. lini* and flax (*L. usitatissimum*) has resulted in formulating the gene-for-gene hypothesis (Flor, 1942). A very comprehensive and detailed study of the pathosystem has distinguished at least 30 interacting *R-Avr* gene pairs and the *R* genes are clustered at five genetic loci (*K*, *L*, *M*, *N* and *P) as* described by Dodds and associates (Dodds et al., 2007). By screening of a flax rust haustorium-specific cDNA library 21 HESPs (haustorially expressed secreted proteins) were identified belonging to four different loci. The avirulence activity was confirmed by expressing *AvrL567*, *AvrM*, *AvrP123* and *AvrP4,* in flax plants carrying the cognate *R* genes. Surprisingly, *AvrM* shows no homology to genes known in public databases and its product contains no cysteine residues in contrast to the proteins AvrP4 and AvrP123 which are cysteine-rich and of which the latter has a Kazal serine protease inhibitor signature (Catanzariti et al., 2006).
The *AvrL567* genes all encode 127 amino acid proteins, are expressed in haustoria and secreted in the extrahaustorial matrix after which they end up in the host cytoplasm. *A. tumefaciens*-mediated transient expression of mature *AvrL567-A* or *AvrL567-B*, but not of *AvrL567-C*, induces an HR in *L. usitatissimum* and *N. tabacum*

during co-expression of the *L5*, *L6*, or *L7* resistance gene, respectively (Dodds et al., 2004). Yeast-two-hybrid assays indicated that Avr567 proteins interact directly with the corresponding R proteins, which could also be confirmed *in planta.* Virulent rust strains escape recognition by altering *Avr* genes, as demonstrated for the *AvrL567* genes which have been examined in detail. Virulent alleles encode proteins with an very high level of amino acid sequence variation (Dodds et al., 2006). Biochemical analyses of heterologously produced AvrL567 protein variants that escape recognition but maintain a conserved structure and stability suggest an important conserved virulence function for these proteins. Similar levels of diversity were observed for the *AvrM*, *AvrP123*, and *AvrP4* genes of flax rust (Catanzariti et al., 2006).

3.5 The *Cladosporium fulvum*-tomato pathosystem

The *Cladosporium fulvum*- tomato (*Solanum lycopersicum)* interaction has been studied intensively by various research groups. From *C. fulvum* (syn. *Passalora fulva*) four race-specific *Avr* genes, *Avr2*, *Avr4*, *Avr4E* and *Avr9*, have been cloned and they all encode small cysteine-rich proteins that are secreted during infection of tomato (Van Kan et al., 1991; Joosten et al., 1994; Westerink et al., 2004 Luderer et al., 2002). Their recognition in tomato is mediated by the cognate Cf (<u>*C*</u>*. <u>f</u>ulvum*) R proteins, Cf-2, Cf-4, Cf-4E and Cf-9, respectively (De Wit et al., 1997; Joosten and De Wit, 1999; Thomma et al., 2005; De Wit et al., 2009). In addition, *C. fulvum* secretes Ecps (<u>E</u>xtra<u>c</u>ellular <u>p</u>roteins) during infection. Ecp1, Ecp2, Ecp4 and Ecp5 have been characterized (Van den Ackerveken et al., 1993b; Laugé et al., 2000) and trigger an HR in tomato accessions that harbor cognate *Cf-Ecp* genes which were all found to map on chromosome 1 close to the *Cf9* homologs (Laugé et al., 1998; Haanstra et al., 1999; Haanstra et al., 2000; Soumpourou et al., 2007). Recently, *Ecp6* and *Ecp7* have been identified but no corresponding *R* genes in tomato accessions have been identified yet (Bolton et al., 2008).

Until recently no homologs of Avr and Ecp proteins have been found in public protein databases, except for Avr4, Ecp2 and Ecp6 which contain orthologs in several other fungal species. While Avr4 possess a chitin-binding motif 14, Ecp6 contains LysM domains which have been associated with binding of various carbohydrates including chitin (Bolton et al., 2008; De Jonge and Thomma, 2009).

Avr4 functions as a virulence factor protecting the fungal cell wall against hydrolysis by plant chitinases (Van den Burg et al., 2003, 2004, 2006; Van Esse et al., 2007). Ecp6 was suggested to be a functional homolog of Avr4 or, alternatively, be involved in sequestering of chitin fragments that are released from fungal cell walls during infection that might otherwise trigger PTI by activating the plant chitin receptor (Miya et al., 2007; Kaku et al., 2006; Bolton et al., 2008; De Jonge and Thomma, 2009).

Also *Ecp1* and *Ecp2* are proven to be involved in virulence (Laugé et al., 1997). Natural and mutant strains lacking or not expressing a functional *Avr9* gene do not

seem to be less virulent on tomato than natural strains harboring a functional *Avr9* gene (Marmeisse et al.,1993) but *Avr9*-(over)expressing tomato plants appear to be more susceptible than control plants to natural *C. fulvum* strains that lack *Avr9*. This suggests that Avr9 is a virulence factor with redundant activity (H. P. van Esse and B. P. H. J. Thomma, personal communication).

3.5.1 The Avr4-Cf-4E interaction

The *Avr4E* gene encodes a small secreted cysteine-rich protein and no biological function besides its recognition by Cf-4E has been reported. The amino acid substitution Phe82>Leu in Avr4E eliminates its recognition by Cf-4E without affecting its stability, which suggests that the amino acid residue Phe82 is important for recognition by Cf-4E (Westerink et al., 2004). Analysis of various strains of *C. fulvum* that can evade Cf-4E-mediated resistance showed that a significant number has jettisoned the *Avr4E* gene or circumvent recognition by point mutations in the *Avr4E* gene without apparent loss of fitness. The virulence function of Avr4E is not yet known, but *Avr4E*-expressing tomato plants are more susceptible than control plants to natural *C. fulvum* strains that lack *Avr4E*, suggesting that Avr4E is a virulence factor (H. P. van Esse and B. P. H. J. Thomma, personal communication).

3.5.2 The Avr4-Cf-4 interaction

The Avr4 protein contains four disulfide bonds (Van den Burg et al., 2001) which contribute to the stability in the protease-rich apoplast of tomato leavrs (Van den Burg et al., 2003; Westerink et al., 2004). Transgenic *A. thaliana* expressing *Avr4* shows an increased susceptibility towards several chitinous fungal pathogens and, similarly, increased susceptibility was observed upon expression of *Avr4* in tomato challenged with the vascular pathogen *Fusarium solani* pv. *lycopersici. Avr4* silencing in *C. fulvum* resulted in decreased virulence on tomato. All these results demonstrate the requirement of Avr4 as an effector for pathogen virulence (Van Esse et al., 2007). Avr4 induces Cf-4-mediated HR, and some natural Avr4 isoforms abolish recognition by Cf-4, but are still able to bind chitin (Van den Burg et al., 2003; Joosten et al., 1997; Stergiopoulos et al., 2007). Heterologous expression of Avr4 *in planta* and injection of purified Avr4 protein triggers Cf-4-dependent HR (Thomas et al., 2000), indicating that binding to fungal chitin is not required for Cf-4-dependent HR. The interaction between Cf-4 and Avr4 is considered to be direct, and this is supported by heterologous Cf-4 expression in many plant species which did not lead to HR autoactivation, but some species activate a necrotic response when Cf-4 is co-expressed with Avr4 (Hammond-Kosack et al., 1998; Thomas et al., 2000; Van der Hoorn et al., 2000; Wulff et al., 2004). Furthermore, several studies have not been able to detect a target in the host plant tomato that could possibly act as a guardee (Westerink et al., 2002; van Esse et al., 2007). Nevertheless, direct binding of Avr4 to Cf-4 could not be experimentally demonstrated thus far.

3.5.3 The Avr9-HABS-Cf-9 interaction

The mature Avr9 peptide contains 28 amino acid residues and its six cysteine residues are all involved in disulfide bonding, creating a cystin-knotted protein that shows structural homology to carboxy peptidase inhibitors and a range of other cystin-knotted inhibitor proteins (Vervoort et al., 1997). However, for Avr9 no inhibitory activity or inhibitor target has yet been identified (Van den Ackerveken et al., 1993a; Van den Hooven et al., 2001; van Kan et al., 1991; Vervoort et al., 1997). A HABS (high-affinity Avr9 binding site) has been detected in microsomal fractions of tomato and other Solanaceous plants, irrespective whether they carried the *Cf-9* gene or not (Kooman-Gersmann et al.,1996). Interestingly, in lettuce Cf-9 did not respond with an HR to treatment with Avr9, most likely due to absence of the HABS (Van der Hoorn et al., 2000). Despite considerable research efforts the HABS has not yet been identified (Van der Hoorn et al., 2001). A systematic mutational analysis of Avr9 has shown a positive correlation between the binding affinity of Avr9 for the HABS and the ability of Avr9 to trigger Cf-9-mediated HR (Kooman-Gersmann et al., 1998). These data suggest that (i) the Avr9-triggered Cf-9-mediated HR requires the HABS, or (ii) that the Avr9-HABS complex activates Cf-9-mediated HR. Notwithstanding extensive research efforts, so far, a direct interaction between the Avr9 protein and the tomato Cf-9 resistance protein could not be proven (Luderer et al., 2001). The *Cf-9* and *Cf-4* genes show similarities and many domain swap and gene-shuffling experiments were performed that have indicated the region of Cf-9 responsible for Avr9 recognition, and the region of Cf-4 responsible for Avr4 recognition, respectively (Van der Hoorn et al., 2001; Wulff et al., 2001). These studies showed that the absence in Cf-4 of two LRRs present in Cf-9 (LRR11 and LRR12) was essential for Cf-4 function but not for Avr4 recognition.

4. Disease resistance genes

After the cloning of *Avr* genes from different microbes the search for their cognate *R*-genes was initiated in many laboratories. The *R* genes and their encoded proteins generally need to meet two basic criteria: they must (i) be involved in perception of the pathogen, and (ii) should subsequently activate defense responses to a level that limits disease progress. Plant R proteins should act as receptors of pathogen Avr proteins and should have a high degree of specificity for pathogen strains. Many *R* genes have been identified since the 1990s. I will discuss only a set of cloned *R* genes for which also the cognate *Avr* genes have been cloned.

4.1 *R* gene cloning and characterization of encoded proteins

The tomato *Pto* gene, the first race-specific *R* gene cloned, provides resistance to particular strains of the Pst bacterium that carry the cognate *avrPto* gene and was isolated by a map-based cloning approach. *Pto* encodes a serine/threonine protein kinase, indicating that it is likely involved in activation of a signal transduction

pathway upon interaction with AvrPto (Martin et al., 1993). *Pto* is very closely linked to the *Fen* gene, encoding another serine/threonine protein kinase, which specifies sensitivity to the fenthion insecticide (Martin et al., 1994). Tomato mutants altered in resistance to the pathogen revealed the presence of an additional gene, *Prf* (*Pseudomonas* resistance and fenthion sensitivity) that is required for both Pto and Fen function (Salmeron et al., 1994). By yeast two-hybrid analysis with Pto as a bait, a second serine/threonine kinase, Pti1 (Pto-interacting 1) was identified, which physically interacts with Pto and may participate in Pto-mediated signaling. Only Pto, but not Fen, phosphorylates Pti1, whereas Pti1 is not able to phosphorylate Pto or Fen. These findings indicate that Pti1 may participate in a downstream Pto-mediated signaling pathway (Zhou et al., 1995).

The tomato *Cf-9* resistance gene, providing resistance against races of *C. fulvum* expressing Avr9, was isolated by transposon tagging. The Cf-9 protein comprises a signal peptide for extracellular secretion and 27 LRRs at the C-terminus, a transmembrane domain and a short cytoplasmic tail (Jones et al., 1994). The cloned *Cf-2* gene, which confers resistance to races of *C. fulvum* that express the cognate *Avr2* gene, encodes a protein with an overall structure that is similar to the Cf-9 protein with the difference that it contains 37 instead of 27 LRRs. Interestingly, the *Cf-2* locus harbors two functional *Cf-2* copies, suggesting a recent gene duplication (Dixon et al., 1996).

The Pst-*A. thaliana* pathosystem was exploited to identify several *R* genes. Here, I will limit myself to the discussion of only two *R* genes, *RPS2* and *RPM1*. The *RPS2* gene was isolated by a map-based cloning approach. A biolistic transformation system was applied for *RPS2* gene delivery into to *rps2* mutants and *RPS2*-mediated HR was demonstrated by infiltration of Pst strains carrying the *avrRpt2* gene (Mindrinos et al., 1994). *RPS2* encodes a 105 kDa protein belonging to the CC-NB-LRR class of cytoplasmic R proteins (Bent et al., 1994). Another CC-NB-LRR-encoding *R* gene is *RPM1,* which confers dual specificity to *P. syringae* expressing *avrB* or *avrRpm1*, two unrelated *Avr* genes. Also the RPM1 protein shares molecular features with reported single-specificity *R* genes. In contrast to all known *R* genes, natural susceptible *A. thaliana* accessions lack any *RPM1* homologs (Grant et al., 1995). Analysis of the whole genome sequence of *A. thaliana* revealed that NB-LRR-encoding genes represent about 1% of the genome and contain on average 14 LRRs with a distinctive 24 amino acid residue repeat length (Meyers et al., 2003). Large-scale expression analyses indicated that alternative splicing occurs in at least 30 *A. thaliana* NB-LRRs (Tan et al., 2007) which can lead to different localizations of the encoded proteins and R proteins lacking or containing only a subset of LRRs (Jordan et al., 2002).

The *A. thaliana RRS1-R* gene that provides resistance against *R. solanacearum* encodes a TIR-NB-LRR and interestingly carries a C-terminal WRKY motif that is characteristic for particular plant transcription factors (Deslandes et al., 2002).

The flax rust resistance gene *L6* contains an NB domain that is similar to other R proteins, but its LRR domain does not fit the canonical LRR structure (Lawrence et al., 1995). The LRR region terminates in two direct repeats, each 146 amino acids in length. Furthermore, the L6 protein shares a domain homologous to the cytoplasmic domain of TIR-NB-LRRs. The *L6* gene can generate four different types of transcripts resulting from alternative splicing (Ayliffe et al., 1999). In total 12 alleles from the flax *L* locus were cloned and all encode TIR-NB-LRRs. Proteins L6 and L11 differ in the LRR region only, but proteins L6 and L7 differ exclusively in the TIR region, indicating that specificity differs between alleles and can be established by both the LRR and the TIR region (Ellis et al., 1999).
Thus, to date three major groups of plant resistance proteins which are activated upon Avr perception have been reported, namely (i) the secreted extracellular cell membrane-attached receptor-like proteins (RLPs), (ii) the intracellular and nuclear CC-NB-LRRs and (iii) the intracellular and nuclear TIR-NB-LLRs. The different domains occurring in most R proteins will be discussed in the following sections.

4.1.1 The LRR domain

The largest domain of most plant R proteins consists of LRRs which are supposed to be involved in protein-protein interactions and might thus also directly or indirectly interact with cognate pathogen Avr proteins. *R*-gene families show a high degree of polymorphism including complete gene duplications which have been evolutionary differentiated by point mutations, deletion(s) and intragenic duplication(s), often including LRRs. The selection pressure imposed on plants by pathogens has forced development of functional resistance specificities as well as maintenance of *R*-gene diversity (Ellis et al., 2000). An LRR unit usually contains a conserved canonical amino acid consensus consisting of Leu-Xxx-Xxx-Leu-Xxx-Leu-Xxx-Xxx-Asn that forms a β-strand followed by a more diverged sequence; each LRR folds into a loop that repetitively forms a stack-like super-helix. The β-strands align on the outside of an arc’s concave surface in a continuous β-sheet. The conserved leucine amino acids face inward to form a stable hydrophobic core. The conserved asparagine amino acids and the variable domains give each repeat a wedge shape-like structure and force a curve-like shape (Kobe and Kajava, 2001). The RLP class of R proteins contains membrane-anchored glycosylated extracellular LRRs (eLRRs) with a well conserved Leu-Thr/Ser-Gly-Xxx-Ile-Pro amino acid motif following the LRR β-strand (Jones and Jones, 1997; Kajava, 1998). Another class of *R* genes encodes NB-LRRs which contain intracellular LRRs (iLRRs) in which the conserved arginine is replaced by a cysteine (Kajava, 1998).

4.1.2 The NB domain

The NB domain is part of the NB-ARC domain, which consists of three sub-domains e.g. NB, ARC1 and ARC2. STAND proteins lack the LRR domain and it has been proposed that they are involved in regulating cellular responses and function as molecular switches through nucleotide-dependent conformational changes (Leipe et al., 2004; Takken et al., 2006). A similar molecular switch function occurs in plant NB-LRR proteins (Tameling et al., 2006). When bound to ATP, the NB-LRR is activated leading to a strong HR. Native ATPase activity brings the NB-LRR into its resting stage. Due to the ability of these R proteins to initiate host cell death, their activity needs to be strictly controlled, meaning that they stay inactive in the absence of a pathogen, but become activated immediately upon pathogen attack. During infection by pathogens their effector molecules modulate intramolecular host targets that are sensed by NB-LRR proteins leading to a quick exchange of ADP by ATP. This implies that an energy-dependent conformational change in R proteins is crucial for their activity. In a study on the tomato NB-LLR I-2 resistance protein that provides resistance against *Fusarium oxysporum* f. sp. *lycopersici* it was shown that the NB domain is able to bind and hydrolyze ATP (Tameling et al., 2002; 2006). Two mutations in the NB domain of I-2 appeared to keep the protein in an auto-active state unable to hydrolyze ATP. This suggests that accumulation of ATP-bound I-2 triggers continuous defense signaling. Since the sequence identity between NB domains of different NB-LRRs is relatively low it is possible that theses domains work somewhat different in different R proteins (Tameling et al., 2006).

4.1.3 TIR and CC domains

The NB-LRR class of R proteins can be subdivided into TIR-NB-LRR and CC-NB-LRR proteins that often show variation in NB domains, dividing them into evolutionary divergent classes (Meyers et al., 1999; Pan et al., 2000). Furthermore, the TIR-NB-LRR group can be further subdivided into two subgroups depending on the presence or absence of a CNL (C-terminal non-LRR domain) (Dodds et al., 2001). Also the CC-NB-LRR group can be further subdivided based on additional N-terminal variations. The SD (Solanaceous domain) is a long extension of the CC domain that has only been found in the *Solanaceae* (Mucyn et al., 2006; Rairdan and Moffett, 2007). The BED (BEAF and DREF) domain, a zinc finger DNA-binding domain, is found in the rice Xa1 resistance protein and in NB-LRRs of poplar (Aravind, 2000; Tuskan et al., 2006). After recognition of the pathogen either the TIR or CC domain becomes involved in initiation of a resistance response as demonstrated by deletion and mutation analyses of the TIR domain of the tobacco N protein that is active towards the tobacco mosaic virus (TMV) (Dinesh-Kumar et al., 2000), and the CC domain of RPS2 that is active towards Pst strains carrying the cognate AvrRpt2 effector (Tao et al., 2000; Tornero et al., 2002). In potato (*Solanum tuberosum*) the CC-NB-LRR protein Rx, which confers resistance to PVX (Potato virus X), carries a

highly conserved Glu-Asp-Val-Ile-Asp amino acid motif in the CC domain that mediates an intramolecular interaction that is dependent on several domains within the remaining part of both the NB and LRR domains of the Rx protein (Rairdan et. al., 2008). A model was proposed where the LRR and CC domains co-regulate the signaling activity of the NB domain in a recognition-specific manner. Similar results were obtained for the flax P resistance protein where loss of function was observed for several truncated versions of the CNL domains suggesting roles of these domains in resistance towards flax rust (Dodds et al., 2001). In plants, the TIR domain is found almost exclusively in NB-LRR proteins and has been proposed to play a signaling role in defense. Reciprocal TIR domain swaps within flax *L* alleles resulted in changes of R protein specificity, ranging from novel specificities to complete loss of specificity (Luck et al., 2000). The TIR-NB-LRR N protein that confers resistance to TMV interacts with the viral p50 helicase domain and N and p50 locate both in the cytoplasm and the nucleus, whereas nuclear localization of N is required for TMV resistance. The TIR domain of N is critical for initial interaction with p50 facilitated by putative host proteins instead of the LRR domains, whereas a second interaction with p50 occurs directly between the NB and LRR domains (Burch-Smith et al., 2007). The TIR-NB-LRR *RPS4* gene encodes a protein conferring resistance to Pst carrying *avrRps4*. Site-directed mutagenesis of the TIR domain of *RPS4* generated not only loss-of-function mutants but also several gain-of function mutants. In addition, amino acid residues outside the TIR domain are also required for defense signaling and HR (Swiderski et al., 2009).

4.1.4 Associations of R proteins with host factors

Mutational analyses in *A. thaliana* have led to the identification of signaling components downstream of R protein activation (Glazebrook, 2001). Some of these components will be discussed here.

EDS1 encodes a protein with homology to lipases (Falk et al., 1999) and is required for TIR-NB-LRR-mediated resistance, whereas NDR1 is required for CC-TIR-NB-LRR-mediated resistance (Aarts et al., 1998). Thus, EDS1 and NDR1 characterize at least two independent *R*-gene mediated plant defense signaling pathways.

Two other components important for R protein function are SGT1 and RAR1. SGT1 is a component of Skp1-Cullin-F-box proteins (SCF) ubiquitin ligases that target regulatory proteins for degradation, a homolog of the yeast ubiquitin ligase-associated protein, that is essential for the function of several *R* genes (Austin et al., 2002; Azevedo et al., 2002). *N. benthamiana* SGT1 homologs are required for N-mediated resistance towards TMV, Rx-mediated resistance towards PVX and Pto-mediated resistance towards Pst expressing *avrPto* (Liu et al., 2002; Peart et al., 2002). *RAR1* (required for Mla12 resistance) is also required for defense pathways mediated by several R proteins (Muskett et al., 2002; Shirasu et al., 1999; Tornero et al., 2002) and encodes a protein with two zinc-binding CHORD (cysteine- and

histidine-rich domains) domains that were first isolated from barley (*Hordeum vulgare*) (Shirasu et al., 1999). Genetic studies in barley revealed that the polymorphic *Mla* (mildew A) locus harbors a large number of functional *R* genes mediating isolate-specific resistance towards the powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*). These genes encode intracellular CC-NB-LRR proteins. Reciprocal domain swaps between Mla1 and Mla6 resulted in the identification of a recognition domain for the cognate fungal Avr proteins AvrMla1 and AvrMla6, respectively, in each of the proteins. Remarkably, Mla1/Mla6 hybrids that confer AvrMla6 recognition exhibited different RAR1 and SGT1 dependency (Azevedo et al., 2002; Shen et al 2003). RAR1 and SGT1 are required for the function of many intercellular R proteins and appear not only crucial for recognition and activation of R proteins upon pathogenic attack, but also for regulation of downstream defense signaling processes. In absence of a pathogen these R-SGT1-RAR1 protein complexes might be in a resting state (Shirasu et al., 2003). A *rar1* mutant plant is compromised for Mla6 but not for Mla1 resistance. Mla1/Mla6 hybrid proteins show different steady state levels which are correlated with their requirement for RAR1, where the RAR1-independent hybrid protein accumulated to higher levels, whereas the RAR1-dependent hybrid protein to lower levels. The LRR domains of RAR1-independent Mla isoforms but not of RAR1-dependent Mla isoforms interact with SGT1 (Bieri et al., 2004).

The cytosolic HSP90 (heat shock protein 90) is abundantly present in both prokaryotes and eukaryotes and is involved in defense signaling pathways (Richter and Buchner 2001). *A. thaliana* HSP90.1 (AtHSP90.1) is required for full RPS2-mediated resistance towards Pst carrying *avrRpt2* (Takahashi et al., 2003), whereas AtHSP90.2 with a mutation in the ATP-binding domain compromises RPM1-mediated resistance towards Pst expressing AvrRpm1 (Hubert et al., 2003). In the HSP90-RAR1-SGT1 interaction, SGT1 and RAR1 act as co-chaperones of HSP90 and assemble with the R protein into a hetero-multimeric protein complex that functions in signaling and regulation of plant disease resistance (Hubert et al., 2003; Shirasu and Schulze-Lefert, 2003; Takahashi et al., 2003).

Over-expression of *R* genes can lead to Avr-independent HR. This phenomenon has been observed for *Pto* in tomato (Tang et al., 1999), *RPS2* in *A. thaliana* (Tao et al., 2000) and *L* in tobacco (Frost et al., 2004) suggesting that R proteins appear to be under tight control and can escape control by chaperones when over-expressed. In a study on RPS4 over-expression in tobacco an AvrRps4-independent HR was induced that required EDS1, SGT1 and HSP90, whereas both the TIR and NB domains of RPS4 were required (Zhang et al., 2004).

During homeostasis and prior to activation, R proteins must be correctly folded and maintained in a recognition-competent state until effectors of pathogen can be recognized leading to a conformational changes, translocation and downstream signaling. It is essential to avoid inappropriate R protein activation which is a threat to

the cell and the strict control of R proteins requires the SGT1-HSP90 chaperone complex that is structurally and functionally conserved in eukaryotes (Shirasu et al., 2009). Complex interactions of chaperone-chaperone and chaperone-NB-LRR domains have been demonstrated by immuno-precipitation assays, where it appeared that the LRR domain is frequently involved in binding. Impairing the chaperone function has a negative effect on resistance protein activation and function, ranging from reduced resting state levels of particular NB-LRRs to complete failure.

4.1.5 Conclusions on Avr and R proteins

Most plant pathogens have developed sophisticated ways to communicate with their host plants, and during evolution plants have developed an efficient innate immune system enabling them to recognize, and defend themselves towards different types of pathogens. Pathogen recognition induces a chain of host defense responses with three major levels of which the lowest represents the most basic form of defense, also referred to as basal defense, followed by induced defense responses known as PTI (PAMP-triggered immunity) and ETI (Effector-triggered immunity). Successful plant pathogens can breach or lower both PTI and ETI with their effectors. PTI is a non-specific type of innate immunity that is operating in nearly all eukaryotic organisms, which involves recognition of PAMPs by PRRs (pattern recognition receptors). ETI is much more specific and is somewhat comparable to the sophisticated adaptive immune system present in higher eukaryotes like animals. ETI involves perception of effectors by *R* gene-encoded receptors or R proteins. Perception of effectors by R proteins can be either direct or indirect.
NB-LRR-containing R proteins function as immune sensors. The LRR domain is used as a module for recognition, regulating protein activation and signal transduction. The LRR domain appears to be highly adjustable in diverse binding specificities to endogenous and exogenous molecules which are a requirement for successful resistance. In case of a pathogen attack, the cognate effector is recognized by the R protein, after which the NB domain functions as a switch for activation of host defenses, regularly resulting in an HR. Inappropriate R protein folding must be avoided and its activation must be tightly controlled, which involves intramolecular LRR interactions between the various domains such as the TIR domain or TIR and CC domains, and by hetero-multimeric protein complexes involving HSP90, RAR1 and SGT1 (Takken and Tameling 2009). Plant R proteins act in multiprotein complexes and to date only a small number of interacting partners have been identified (Padmanabhan et al., 2009). Unraveling the action of the chaperone machinery has strongly increased our understanding of the mechanisms of pathogen recognition and signal transduction by NB-LRR proteins in both plants and animals.

5. General concepts: the Zig-Zag model

For quite some time, plant pathogen researchers have pondered about the intrinsic functions of pathogen Avr factors. Indeed their sole function is very unlikely to be allowing the host to recognize the pathogen. In the late 1990s it was shown that Avr factors function as virulence factors in the absence of cognate R proteins. Many interact with host virulence targets in order to suppress basal defense, PTI or ETI in order to promote disease. Specific host R proteins often function as guards of these virulence targets and, upon modulation of a host virulence target by Avrs, a quick defense response is initiated often associated with an HR (Dangl and Jones, 2001; Dixon et al., 2000; Mackey et al., 2003). This scenario is very well explained by studying the Pst- *A. thaliana* interaction in great detail and has revealed that during evolution plant bacterial pathogens have developed various and sophisticated virulence factors (besides toxins, enzymes, hormones and polysaccharides) that are injected in the host by the TTSS. These virulence factors are employed for the suppression of plant immunity to enable the pathogen to stimulate, or sometimes force, the host to release nutrients into the apoplast. However, plants have evolved an efficient innate immune system enabling them to recognize, and defend themselves towards different types of bacterial and fungal pathogens. The co-evolutionary model explaining host pathogen interactions at all levels is comprised in the so-called Zig-Zag model proposed by Jones and Dangl (2006) (Figure 2).

In brief, microbes, including nonpathogenic ones, carry PAMPs or MAMPs that are recognized by the host surveillance system which consist of cell surface receptor proteins that activate a set of anti-pathogen defense mechanisms. Via mitogen-activated protein kinases (MAPKs) and hormone signaling pathways this recognition leads to PAMP-triggered immunity (PTI) (Chisholm et al., 2006; Jones and Dangl, 2006).

Successful plant pathogens can breach PTI through secretion of specific effector molecules which enables them to colonize the host; this phenomenon is called ETS (effector-triggered susceptibility). Host plants have responded to ETS by the development, during co-evolution, of effector receptors or R proteins enabling them to respond to effectors by cognate R-mediated defense associated with an HR at the site of infection. In addition, effectors might undergo changes in order to escape recognition by their cognate resistance proteins leading to a continuous arms race between pathogen and host plant. This arms race between pathogen and host plant can continue for ages and has led to development of numerous effectors and as many cognate R proteins. Perception of effectors by R proteins can be either direct or indirect which, has also been the subject of my thesis with emphasis on the interaction of Cf-2 with Avr2 which requires the Rcr3 cysteine protease (Krüger et al., 2002).

6. Outline of thesis

As discussed above, the outcome of the interaction between many (hemi)biotrophic pathogens and their host plants has been defined by presence or absence of host R proteins and their cognate pathogen effectors. Usually pathogen effectors facilitate pathogen growth by means of suppression of host defenses and manipulation of host metabolism. This manipulation of the host leads to cognate R-mediated recognition and defense that enables the host to limit pathogen growth and eradicate the pathogen. This indirect recognition of a pathogen effector through its manipulation of the R protein-guarded virulence target is also known as the guard model which has been proposed initially by van der Biezen and Jones (1998) and reviewed by Dangl and Jones (2001) (Figure 2). This model is in contrast to direct interaction between effector and R protein, as initially was supposed to occur for all effector-R protein interactions, as deduced from Flor's gene-for-gene hypothesis. In contrast to many other biotrophic fungal pathogens, *C. fulvum* is an extracellular fungal pathogen that thrives in the apoplastic space without producing haustoria in host cells to retrieve nutrients. This specific way of life of this pathogen of tomato has enabled us to isolate important host and fungal factors acting in this apoplastic arena. It has enabled us to isolate 10 fungal effectors, and for most of them cognate host R proteins have been identified as well.

These effectors are thought to act in avoiding or suppressing basal host defense, and they are all small, and cysteine-rich proteins, but otherwise share no homology. They include Avr2, Avr4, Avr4E, Avr9, and six Ecps (extracellular proteins) that have been discussed before. An intrinsic function for two effectors has been described including Avr4, a chitin binding protein that protects the fungus against the deleterious effects of tomato chitinases, and Avr2 which is the subject of this PhD thesis.

Chapter 1 presents an overview of well-studied gene-for-gene interactions and discusses direct and indirect interactions between pathogen effectors and cognate host resistance proteins (Figure 3).

In **Chapter 2** we describe the avirulence function of Avr2 in detail and show that this effector inhibits the cysteine protease Rcr3 (required for *C. fulvum* resistance) which triggers Cf-2-mediated defense responses including an HR. We also show that inhibition of Rcr3 is probably not crucial for triggering of Cf-2-mediated HR, as inhibition of Rcr3 by the irreversible cysteine protease inhibitor E-64 does not trigger Cf-2-mediated HR. Thus, in this chapter we have, for the first time, provided evidence for the existence of a virulence target of a fungal effector of which the modulation by the effector is sensed by the cognate R protein. As such it is the first evidence for the existence of the guard model in a fungal pathosystem. The physical interaction between the cysteine protease Rcr3 and the fungal effector Avr2 was studied in detail by cysteine protease activity profiling assay, using the biotinylated irreversible cysteine protease inhibitor E-64 (DCG-04). This assay could be used *in vitro* as well

as *in planta* showing that the interaction was biologically relevant. We could show the presence of the Avr2-Rcr3 complex by co-immunoprecitation assays. Biological relevance of Avr2 and Rcr3 *in planta* was also examined by injection of both proteins in *Cf-2/rcr3* tomato leaves (lacking a functional Rcr3), showing that only these two proteins are responsible for activation of the Cf-2 resistance protein and no other Avr2-cysteine protease complexes.

In **Chapter 3**, the intrinsic virulence function of Avr2 was studied. Detailed cysteine protease profiling revealed that Avr2 specifically interacts with several cysteine proteases including not only Rcr3 but also Pip1, aleurain and TDI65. Thus during infection Avr2 plays an offensive role in virulence by targeting and inhibiting host proteases that are important for host defense. Heterologous (over)expression of *Avr2* in tomato was found to promote colonization of race 2 *C. fulvum* strains, while silencing of *Avr2* in a race 5 *C. fulvum* strain compromised virulence on tomato. Heterologous expression of *Avr2* in *A. thaliana* enhanced susceptibility towards the fungal pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina.* Furthermore cysteine protease profiling in *A. thaliana* revealed that Avr2 specifically inhibits certain families of extracellular *A. thaliana* cysteine proteases which correlates with the data obtained for tomato.

Chapter 4 describes the *C. fulvum* Avr2 effector as a novel type of cysteine protease inhibitor and shows that the eight cysteines present in Avr2 are all involved in disulfide bonds. We produced wild type Avr2 protein in *Pichia pastoris* and determined its disulfide bond pattern. By site-directed mutagenesis of all eight cysteine amino acids we show that three of the four disulfide bonds are required for Avr2 stability. We show that the six C-terminal amino acids of Avr2 contain a disulfide bond that is not embedded in the overall Avr2 structure. We also show that Avr2 is not processed by the tomato cysteine protease Rcr3 and behaves as an uncompetitive inhibitor. Subsequently, we produced mutant Avr2 proteins in which selected amino acids were individually replaced by an alanine residue, or all six C-terminal amino acids were deleted. Of these Avr2 mutants we determined their affinity (K_i) for Rcr3 and their ability to trigger a Cf-2-mediated HR in tomato. We found that the two C-terminal cysteine amino acids and the six amino acid C-terminal tail of Avr2 are required for Rcr3-inhibitory activity and the ability to trigger a Cf-2-mediated HR. Overall, our data suggest that the affinity of the Avr2 mutants for Rcr3 is positively correlated with their ability to trigger a Cf-2-mediated HR.

In **Chapter 5** I discuss the results obtained in a broader perspective. I start with an overview of different plant cysteine proteases involved in plant development and plant defense. Also a summary of microbial pathogen cysteine proteases and cysteine protease inhibitors involved in host-pathogen interactions is provided. I then return to our own results and put them in a broader perspective by discussing them in relation to findings by other colleagues in recent years. I subsequently provide suggestions for future research on the Avr2-Rcr3-Cf-2 complex and its putative interacting proteins which compose the so-called resistasome.

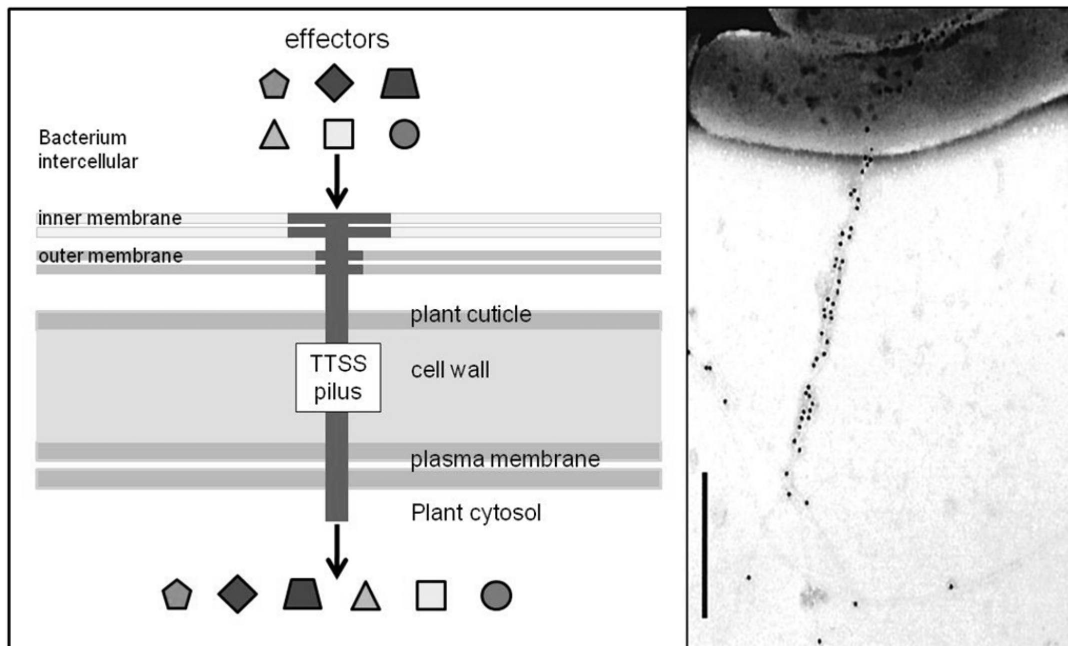

Figure 1. Schematic representation of the bacterial type three secretion system (TTSS).
Left; the TTSS pilus consists of a double bacterial membrane, which extends and passes the plant cell wall and plasma membrane. Effectors are delivered through the pilus from the bacterial cytoplasm to the host cytosol where they are often involved in suppression host plant defense. Right; the *hrpA* mutant of *Pseudomonas syringae* pv. *tomato* (Pst) expressing FLAG-HrpA showing the presence of Hrp-A in the pilus 18 hours after incubation in Hrp-inducing medium. Immuno-gold localization with anti-FLAG antibody shows that the TTSS pilus is nearly entirely composed of FLAG-HrpA (Li et al., 2002). (Bar = 0.5 μm).

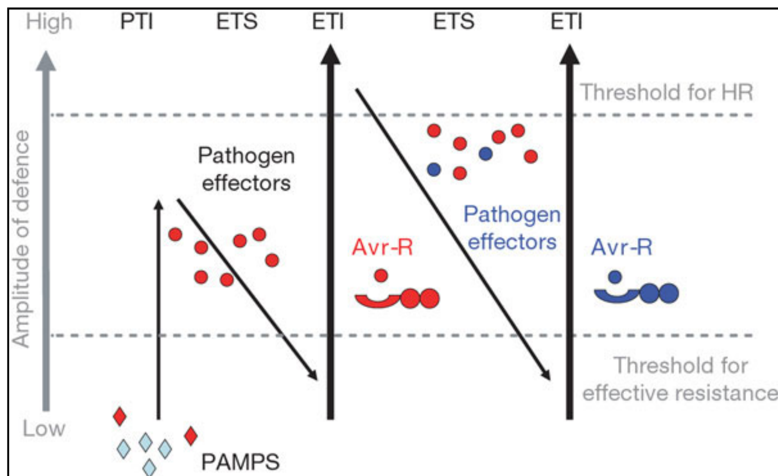

Figure 2: The current model of the plant immune system: the Zig-Zag model
(after Jones and Dangl, 2006).
From left to the right:

1. **PTI** (PAMP-triggered immunity) represents a basal host plant defense response mediated by PRRs (pathogen recognition receptors) after recognition of PAMPs (pattern-associated molecular patterns ( ).
2. **ETS** (Effector-triggered susceptibility) describes the phenomenon where successful pathogens produce (secrete) effectors ( ) that can suppress or mitigate PTI.
3. **ETI** (Effector-triggered immunity) describes the phenomenon where an effector of the pathogen (filled red circles) is recognized by a cognate R protein ( ) that subsequently mediates the induction of a defense response often involving a hypersensitive response (HR) at the site of the infection; the effector has now become an avirulence factor (Avr)
4. This is the start of the so-called arms race between pathogen and plant that leads to adaptation of effectors that avoid recognition by cognate R proteins or acquiring new effectors ( ) that can suppress **ETI**; selection will drive to development of new R proteins( ) to recognize newly acquired effectors resulting again in **ETI.**

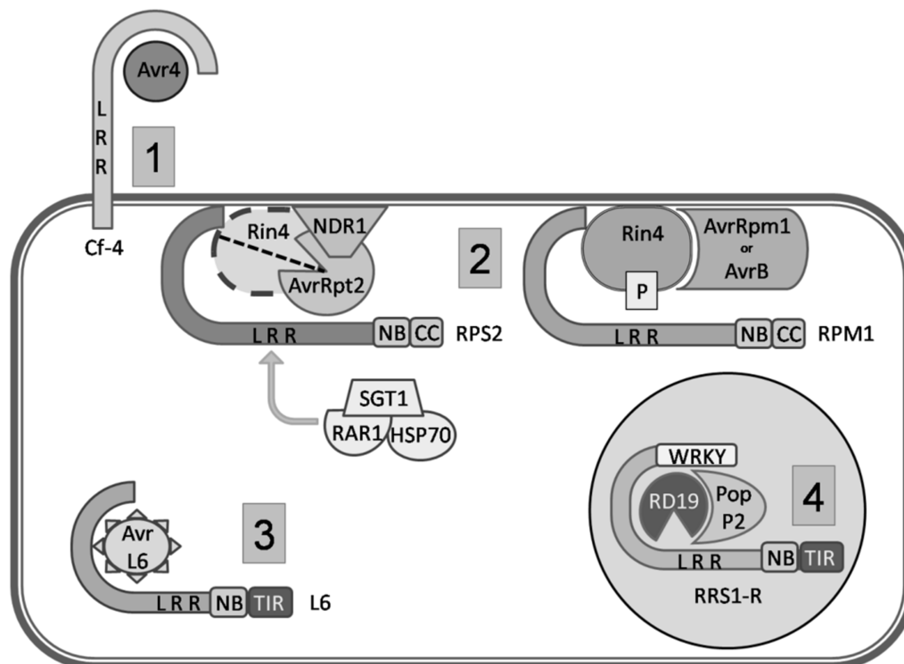

Figure 3: Schematic overview of different types of direct and indirect interactions between bacterial and fungal effectors with their plant virulence targets and/or cognate R proteins.
1. An example of extracellular LRR R protein such as tomato Cf-4 that is thought to interact directly with the cognate *C. fulvum* effector protein Avr4. 2. Examples of intracellular CC-NB-LRR type of R proteins (RPS2 and RPM1) that interact indirectly (through Rin4), with their cognate *Pseudomonas syringae* effector proteins AvrRpt2, AvrRpm1 or AvrB, respectively. 3. An example of direct interaction between an intracellular TIR-NB-LRR type of R protein (L6) of flax (*Linum usitatissimum*) with AvrL6 of the flax rust fungus (*Melampsora lini*). 4. An example of a nuclear TIR-NB-LRR type of R protein (RRS1-R) from *Arabidopsis thaliana* showing direct interaction with the *Ralstonia solanacearum* PopP2 effector that also requires vacuolar cysteine protease RD19.

LRR: leucine-rich repeat
NB: Nucleotide Binding site
CC: Coiled Coil
TIR: Toll and interleukin receptor
WRKY: DNA binding motif containing Trp-Arg-Lys-Tyr residues
P: Phosphorylation
Avr4: Avirulence protein 4 from *C. fulvum*
Cf-4: Resistance protein to *C. fulvum* 4
AvrRpm1: Avirulence protein from *Pseudomonas syringae* pv. *maculicola* 1
AvrRpt2: Avirulence protein from *Pseudomonas syringae* pv. *tomato* 2
AvrB: Avirulence protein B from *Pseudomonas glycinea*
Rin4: Rpm1-interacting protein 4
NDR1: nonspecific disease resistance 1
RPS2: Resistance protein against *Pseudomonas syringae* 2
RPM1: Resistance protein against *Pseudomonas syringae* pv. *maculicola* 1
RAR1: Required for Mla12 resistance 1
HSP90: Heat shock protein 90
SGT1: Suppressor of g2 allele of skp1
RD19: Responsive to dehydration-19
AvrL6: Avirulence protein L6 from *Melampsora lini*
L6: Resistance protein L6
PopP2: *Pseudomonas solanacearum* outer protein P2
RRS1-R: Resistance to *Ralstonia solanacearum*1- R

CHAPTER 2

Cladosporium Avr2 inhibits tomato Rcr3 protease required for Cf-2-dependent disease resistance

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Abstract

How plants recognize pathogens and activate defense is still mysterious. Direct interaction between pathogen avirulence (Avr) proteins and plant disease resistance (R) proteins is the exception rather than the rule. During infection, *Cladosporium fulvum* secretes Avr2 protein into the apoplast of tomato leaves and, in the presence of the extracellular LRR receptor-like Cf-2 protein, triggers a hypersensitive response (HR) that also requires the extracellular tomato cysteine protease Rcr3. We show here that Avr2 binds and inhibits Rcr3, and propose that the Rcr3-Avr2 complex enables the Cf-2 protein to activate an HR.

Introduction

Plant disease resistance (*R*) genes mediate race-specific recognition of pathogens via perception of avirulence (*Avr*) gene products (Flor, 1971). Tomato (*Lycopersicon esculentum*) *Cf* genes confer resistance to leaf mould caused by *Cladosporium fulvum*, and encode transmembrane receptorlike proteins (RLPs) with extracellular leucine-rich repeats (LRRs) that mediate recognition of fungal Avrs secreted during infection (Joosten and de Wit, 1999). *Cf*-dependent perception of Avrs activates plant defense, including the hypersensitive response (HR), which results in host cell death at the site of penetration and limits pathogen ingress (de Wit, 1977; Lazarovits and Higgins, 1976). How Cf proteins enable tomato to perceive Avrs is unknown. So far, no direct interaction between Cf proteins and Avr proteins has been detected (Luderer, 2001). So far, a direct interaction has only been demonstrated for two LRR-containing R-Avr pairs (Deslandes, et al., 2003; Ron and Avni, 2004). The lack of a direct interaction led to the formulation of the guard hypothesis (van der Biezen and Jones, 1998; van der Hoorn, et al., 2002), proposing that Avrs are virulence factors that interact with host targets to facilitate pathogen growth in the host, and that R proteins monitor the status of these host targets (Chang, et al., 2004). Cf-*2* confers resistance to *C. fulvum* in tomato (Dixon, et al., 1996) based on perception of Avr2, a cysteine-rich protein secreted by the fungus (Luderer, et al., 2002). Cf-2 function also requires Rcr3 (Dixon, et al., 2000), a secreted tomato cysteine protease (Krüger, et al. 2002), which is not required by other *Cf* genes, including the highly homologous *Cf-5* gene (Dixon, et al., 2000; Krüger, et al. 2002). The *L. esculentum* allele encodes the Rcr3^{esc} protein that weakly activates *Cf-2*-dependent HR in tomato leaves in absence of Avr2. The *L. pimpinellifolium* allele encodes Rcr3^{pim} required for Cf-2 to confer an Avr2 response (Krüger, et al. 2002). We hypothesized that Rcr3 is a target of Avr2.

Results

To test this hypothesis, we produced Rcr3 as a C-terminal 6xHistidine (His)- and haemagglutinin (HA)-tagged protein fusion (Rcr3-His-HA) both in *Nicotiana benthamiana* and in *Pichia pastoris*. Mature Rcr3 was recovered from intercellular fluid (IF) of *N. benthamiana* leaves using the tags on the fusion protein (Krüger, et al., 2002).

To monitor Rcr3 activity, we applied protease activity profiling at pH 5 using DCG-04, a biotinylated derivative of the irreversible cysteine protease inhibitor E-64, which has been used to profile cysteine protease activities from mammals (Greenbaum, et al., 2000), insects (Kocks, et al., 2003), and plants (van der Hoorn, et al., 2004). DCG-04 treatment leads to irreversible labelling of cysteine proteases with biotin. Labelling of Rcr3 with 220 nM DCG-04 was assayed in the presence or absence of different concentrations of E-64 as a competitive inhibitor. After reaction with DCG-04, Rcr3-His-HA was pulled down using Ni-NTA (binding to His-tag) or streptavidin (binding to biotin) beads. In the absence of E-64, DCG-04 biotinylates Rcr3, confirming that Rcr3 is a cysteine protease, whereas in the presence of 1140 nM E-64, Rcr3 is not biotinylated (Fig. 1A).

We tested if Avr2 could inhibit biotinylation of Rcr3 by DCG-04. As a negative control *C. fulvum* Avr4, which triggers *Cf-4*-dependent HR (Joosten, et al., 1994), was included. Both Avrs were expressed in *P. pastoris* as N-terminal His-FLAGfusions and purified on a Ni-NTA column. In the presence of 140 nM Avr2, Rcr3 is not biotinylated (Fig. 1A) indicating that Avr2 inhibits Rcr3 activity (see fig. S1 for titration curve). In the presence of Avr4, Rcr3 is biotinylated, showing that inhibition of Rcr3 by Avr2 is specific (Fig. 1A and fig. S1).

Intercellular fluid (IF) obtained from tomato has a pH of approximately 5 (Grignon, et al.,1991).To investigate the pH-dependence of Rcr3 activity and its inhibition by Avr2, *N. benthamiana* IF containing Rcr3 was incubated with DCG-04 in the absence or presence of an excess of E-64 or Avr2 over a pH range of 4.5-6.5. Rcr3 activity is highest at pH 5-6, and strongly decreases outside this range (Fig. 1B). Inhibition by E-64 is effective over the whole pH range, whereas inhibition by Avr2 is only effective below pH 6 (Fig. 1B), indicating that the pH optimum for Rcr3 activity and its inhibition by Avr2 coincide with the pH of the apoplast of tomato (pH 5).

Rcr3 produced as a C-terminal His-HA-fusion in *P. pastoris* is also inhibited by E-64 and Avr2 (Fig. 1C), indicating that Avr2 alone is sufficient to inhibit Rcr3 and that no additional plant factors are required. No biotinylation by DCG-04 of other cysteine proteases was observed in control IF from *N. benthamiana* non-transgenic for Rcr3 (Fig. 1A) or control culture supernatant (CS) from *P. pastoris* non-transgenic for Rcr3 but expressing Ecp4, another protein secreted by *C. fulvum* (Laugé, et al., 2000) (Fig. 1C). This indicates that no endogenous extracellular cysteine proteases were biotinylated at the DCG-04 concentration used (220 nM).

Since Avr2 inhibits Rcr3, we expected a physical interaction between the two proteins. This was investigated by co-immunoprecipitation studies. His-FLAG-Avr2 or His-FLAG-Avr4 was added to *N. benthamiana* IF or culture supernatant (CS) of *P. pastoris* containing Rcr3-His-HA, and immunoprecipitated with a FLAG-specific antibody (α- FLAG). As a control, Rcr3-His-HA was also pre-incubated with E-64 to block the active site before adding His-FLAGAvr2. After immunoprecipitation, the samples were run on SDS gel, blotted and Avr proteins and Rcr3 were detected using α-FLAG and an Rcr3-specific antibody (α-Rcr3), respectively (Fig. 2). Rcr3 co-immunoprecipitates with Avr2 but not with Avr4 (Fig. 2), indicating a specific interaction between Avr2 and Rcr3. Blocking the active site of Rcr3 eliminates this interaction (Fig. 2). In the presence of Avr2, α-FLAG co-immunoprecipitates Rcr3, irrespective of the source of Rcr3, again indicating that the interaction between Avr2 and Rcr3 is independent of additional plant factors (Fig. 2). No signals were detected on blots probed with α-Rcr3 after immunoprecipitation of proteins from control *N. benthamiana* IF (marked pBin19; Fig. 2) or control CS of *P. pastoris* non-transgenic for Rcr3 (marked Ecp4; Fig. 2), indicating that α-Rcr3 is specific.
We tested whether native Rcr3 in tomato IF can be detected and inhibited by E-64 and Avr2. Six ml of IF from different Cf tomato plants producing Rcr3 were brought to 2.2 μM DCG-04 in the presence or absence of E-64 (28.6 μM) or Avr2 (6.9 μM). Biotinylated proteins were selected with streptavidin beads, run on an SDS gel and probed with α-Rcr3 or streptavidin-HRP (fig. S2). Native Rcr3 is detected by α-Rcr3 in Cf0 and Cf2 tomato lines, and its biotinylation by DCG-04 is inhibited by Avr2, whereas in *Cf-2/rcr3-3* plants (Dixon, et al., 2000; Krüger, et al., 2002) Rcr3 is absent (fig. S2; upper panel). In addition to Rcr3, several other apoplastic cysteine proteases are biotinylated that can be inhibited by Avr2 (fig. S2; lower panel).
To determine whether inhibition of Rcr3 by Avr2 is sufficient to trigger *Cf-2*-dependent HR, Rcr3 produced in *N. benthamiana,* either alone or in combination with Avr2, E-64 or E-64 and Avr2, or Avr2 alone, was infiltrated into *Cf- 2*/*rcr3-3* tomato leaves (Fig. 3). Infiltration of Avr2 or Rcr3 alone, or Rcr3 incubated with E-64 does not trigger an HR, whereas infiltration of Rcr3 incubated with Avr2 does. However, Rcr3 pre-incubated with an excess of E-64 to saturate the active site, and subsequently incubated with Avr2, does not trigger *Cf-2*-mediated HR. Similar results were obtained with *P. pastoris*-produced Rcr3 pre-treated with the same compounds (results not shown).

In summary, Avr2 inhibits tomato Rcr3 in a pH-dependent fashion. Avr2 binds to Rcr3 produced in *N. benthamiana* or *P. pastoris*, showing that no additional plant factors are required for binding and inhibition. Native tomato Rcr3 is detected by α-Rcr3 and inhibited by Avr2. In addition, other native tomato cysteine proteases are inhibited by Avr2. Finally, co-infiltration of Rcr3 pre-incubated with E-64 and Avr2 blocks *Cf-2*-dependent HR in *Cf-2/rcr3-3* tomato, demonstrating that the Rcr3-Avr2 complex is required for the initiation of the HR.

Inhibition of Rcr3 activity by Avr2 could either be due to Avr2 acting solely as an inhibitor of Rcr3, or Avr2 being both a substrate and an inhibitor. However, we observed no degradation of Avr2 upon incubation with Rcr3 (Fig. 2), suggesting that Avr2 is not a substrate for Rcr3. Furthermore, if processing of Avr2 by Rcr3 were required for *Cf-2*- mediated HR, then Avr2 present in IF from Cf0 tomato plants (containing Rcr3), infected by Avr2-producing *C. fulvum* strains, would induce an HR in *Cf-2/rcr3-3* tomato. This was not observed (results not shown), indicating that Avr2 is an inhibitor, not a substrate, of Rcr3. However, inhibition of Rcr3 activity is not sufficient to initiate Cf-2-mediated HR (Fig. 3). Therefore we propose that inhibition of Rcr3 by Avr2 induces a conformational change of Rcr3 that triggers the Cf-2 protein to activate HR. This model is consistent with the observation that the $Rcr3^{esc}$ protein alone provokes a weak *Cf-2*-dependent, Avr2-independent HR (Krüger, et al., 2002). We suggest that $Rcr3^{esc}$, which differs from $Rcr3^{pim}$ in one amino acid deletion and six amino acid changes, constitutively mimics the conformational change imposed on $Rcr3^{pim}$ (present in *Cf-2* plants) by Avr2 binding, and weakly activates *Cf-2*-dependent HR in absence of Avr2. The role of Rcr3 cysteine protease activity for tomato and the importance of its inhibition by Avr2 for *C. fulvum* during infection are unknown, but possibly secreted plant cysteine proteases have anti-microbial activity. *Rcr3* transcription is induced faster and transcripts accumulate to higher levels in incompatible compared to compatible interactions between tomato and *C. fulvum* (Krüger, et al., 2002), as do transcripts for pathogenesisrelated proteins after infection by this fungus (van Kan, et al., 1992). Rcr3 is also induced in the absence of Cf-2, consistent with a role for Rcr3 in basal host defense (Krüger, et al., 2002). Furthermore, in addition to Rcr3, several other apoplastic cysteine proteases are inhibited by Avr2 suggesting that Avr2 is a general virulence factor facilitating growth of *C. fulvum* in the apoplast.

Recently, it has been shown that a protease inhibitor from *Phytophthora infestans* interacts with and inhibits the plant serine protease P69B, which is induced during infection of tomato by this pathogen (Tian, et al., 2004). Thus, inhibition of plant proteases may represent a general counter-defense employed by invading pathogens. The role of Rcr3 in the perception of Avr2 by Cf-2 is consistent with the guard hypothesis. The Rcr3-Avr2 complex, but not other Avr2-cysteine protease complexes, activates Cf-2. So far, all bacterial pathogens colonizing the apoplast of plants deliver their effector proteins into the plant cell by the type-III secretion system

where they interact with cytoplasmic virulence targets (Chang, et al., 2004; Axtell, et al., 2003a; Axtell, et al., 2003b; Mackey, et al., 2002; Mackey, et al., 2003). In the case of RPM1- and RPS2-mediated resistance in Arabidopsis, the action of the Avr proteins AvrB, AvrRpm1 and AvrRpt2 on the "guardee" RIN4 is thought to trigger the activation of the RPM1 or RPS2 proteins (Axtell, et al., 2003a; Axtell, et al., 2003b; Mackey, et al., 2002; Mackey, et al., 2003). Similarly, in RPS5- mediated resistance in Arabidopsis the cysteine protease activity of the Avr protein, AvrPphB, on the guardee PBS1 is required to trigger the HR (Shao, et al., 2003). Such indirect interactions between pathogen Avrs and plant R proteins may be more difficult for the pathogen to circumvent without a virulence penalty than direct interactions (van der Biezen and Jones, 1998; van der Hoorn, et al., 2002). In addition to Rcr3, other tomato cysteine proteases are inhibited by Avr2 that are not guarded by known Cf proteins. Characterization and functional analysis of these proteases will be subject of future studies.

Notes

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Figures and figure legends

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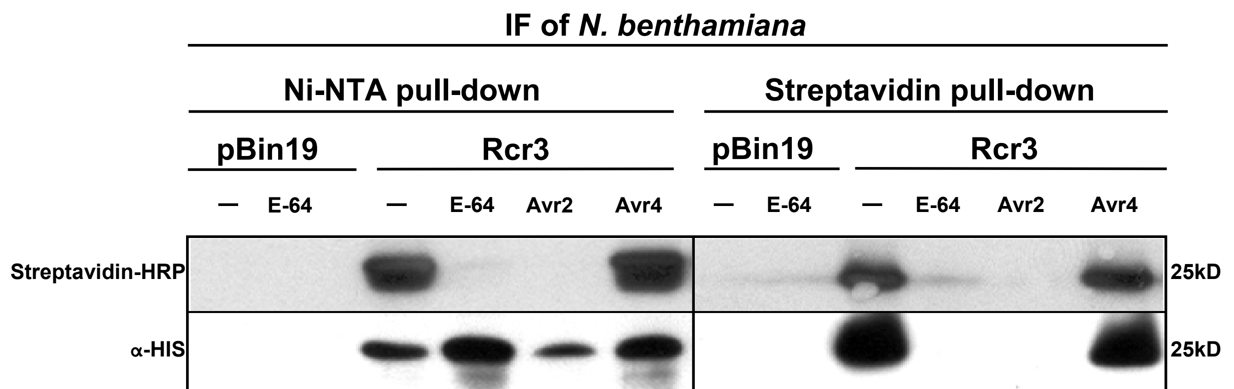

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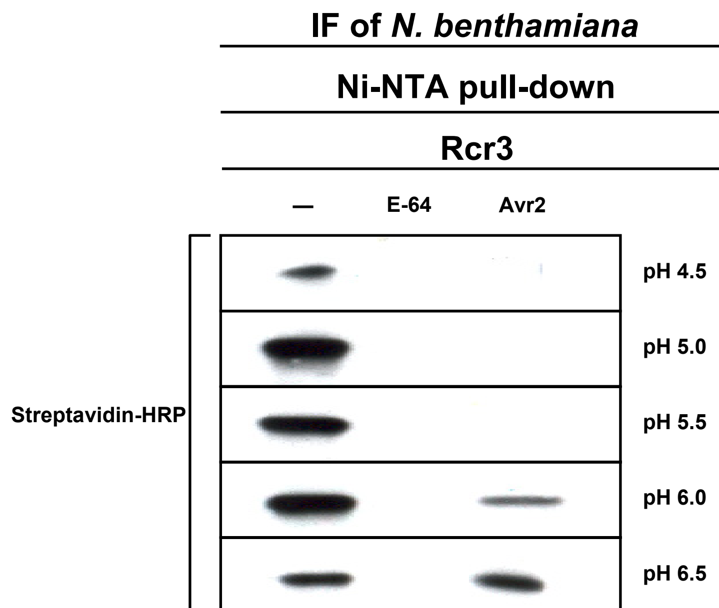

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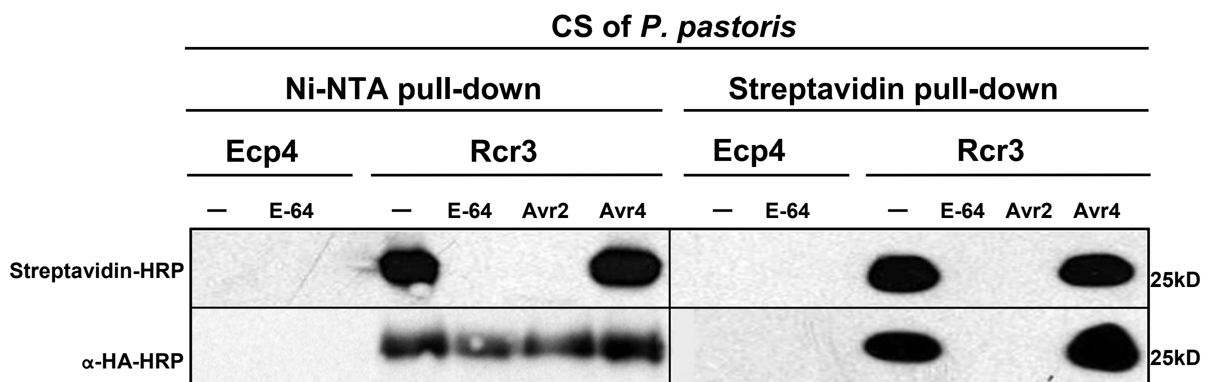

Fig. 1. The Rcr3 cysteine protease of tomato is inhibited by Avr2 of *C. fulvum*.

(**A**) Inhibition of Rcr3 produced in *Nicotiana benthamiana* by Avr2. Intercellular fluid (IF) was isolated from *N. benthamiana* expressing either the empty vector (pBin19) or Rcr3-His-HA (Rcr3). Protease activity profiling with 220 nM DCG-04 was performed in the absence of inhibitor (-), or in the presence of E-64, His-FLAG-Avr2 (Avr2) or His-FLAG-Avr4 (Avr4). Rcr3-His-HA was pulled down by Ni-NTA beads (left panels) or by streptavidin beads (right panels), electrophoresed on an SDS gel and immunodetected with streptavidin-HRP or His-specific antibodies (α- His). Detection with streptavidin-HRP reveals that Rcr3 is not biotinylated in the presence of E-64 or Avr2, whereas biotinylation of Rcr3 occurs without inhibitor or with Avr4, indicating that like E-64, Avr2 inhibits Rcr3 cysteine protease activity. α-His always detects Ni-NTA-pulled down Rcr3-His-HA irrespective of whether Rcr3 is inhibited or not (left panels), whereas α-His only detects biotinylated Rcr3-His-HA when Rcr3 is not inhibited by E-64 or Avr2 (right panels marked – and Avr4). No cysteine proteases are biotinylated in the empty vector control (pBin19). (**B**) Inhibition of Rcr3 by Avr2 is pH-dependent. IF from *N. benthamiana* containing Rcr3-His-HA was profiled with 220 nM DCG-04 in the absence of inhibitor (-), and in the presence of E-64 (1120 nM) or Avr2 (140 nM), over a pH range from 4.5 to 6.5. Rcr3-His-HA was pulled down by Ni-NTA beads and immunodetected with streptavidin-HRP to demonstrate biotinylation. Rcr3 is biotinylated in the absence of inhibitor (-), with highest levels of biotinylation between pH 5.0 and 6.0. Inhibition of biotinylation of Rcr3 by E-64 is complete at all pH values, whereas inhibition by Avr2 decreases at pH values above 6.0. (**C**) Inhibition of Rcr3 produced in *Pichia pastoris* by Avr2. Culture supernatant (CS) was isolated from *P. pastoris* cultures expressing either His-FLAG-Ecp4 (Ecp4) or Rcr3-His-HA (Rcr3) and protease activity was profiled with 220 nM DCG-04 in the absence of inhibitor (-), in the presence of E-64 , His-FLAG-Avr2 (Avr2) or His-FLAG-Avr4 (Avr4). Subsequently, Rcr3 was pulled down by Ni-NTA beads (left panels) or by streptavidin beads (right panels), electrophoresed on an SDS gel and immuno-detected with streptavidin-HRP or HA-specific antibodies (α-HA-HRP). Detection with streptavidin- HRP reveals that in the presence of E-64 or Avr2, Rcr3 is not biotinylated, whereas biotinylation of Rcr3 occurs in the absence of inhibitor or in the presence of Avr4, indicating that similar to E-64, Avr2 inhibits Rcr3 cysteine protease produced in *P. pastoris* as does Rcr3 produced in *N. benthamiana.*

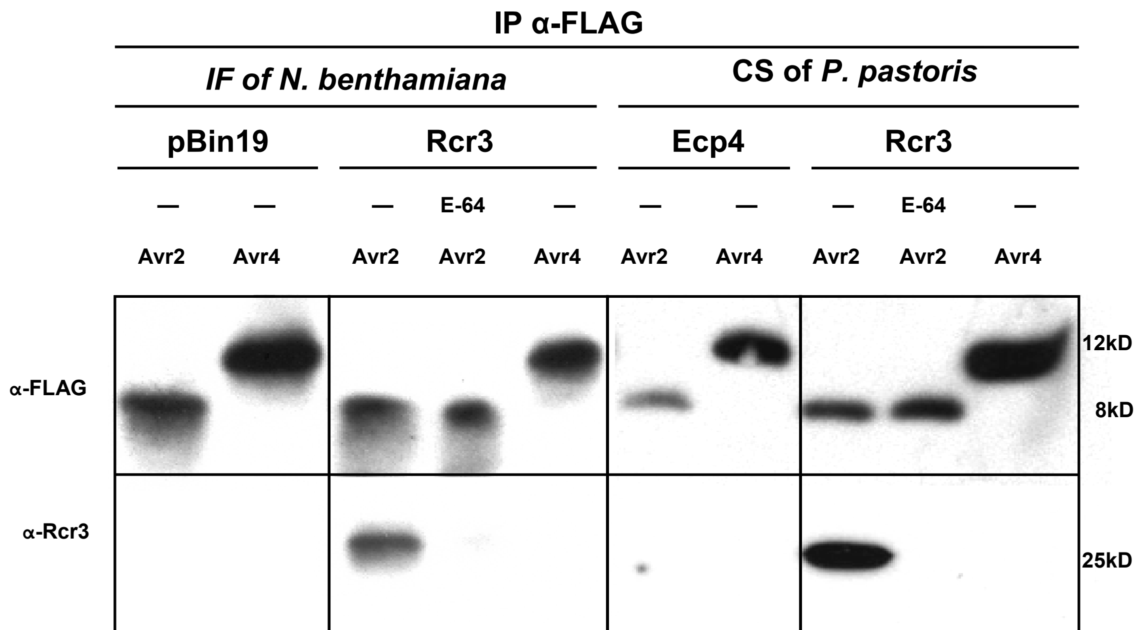

Fig. 2. Rcr3 and Avr2 physically interact.
Rcr3-His-HA (Rcr3) was incubated with His-FLAG-Avr2 (Avr2), with or without pre-treatment with E-64, or His-FLAG-Avr4 (Avr4) and then immuno-precipitated with FLAG-specific antibody (α-FLAG). IF from *N. benthamiana* expressing either the empty vector (pBin19) or Rcr3, or CS from *P. pastoris* expressing either His-FLAG-Ecp4 (Ecp4) or Rcr3, was incubated without inhibitor (-) or with an excess of E-64 before adding Avr2 or Avr4. After immuno-precipitation with α-FLAG beads, the Avr proteins were detected with α-FLAG, whereas co-immunoprecipitated Rcr3 was detected with α-Rcr3. Rcr3 is only co-immunoprecipitated in the presence of Avr2 without pre-incubation with E-64.

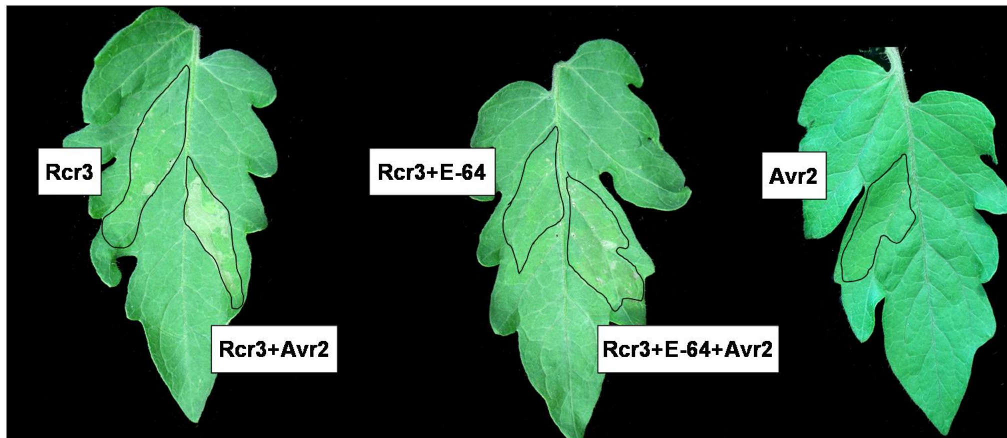

Fig. 3. Cf-2-mediated HR requires physical interaction between Rcr3 and Avr2.
Fully expanded leaves of 5-week-old *Cf-2/rcr3-3* tomato were infiltrated with Rcr3-His-HA (Rcr3), produced in *N. benthamiana*, either alone or in combination with His-FLAG-Avr2 (Avr2), E-64 or E-64 and Avr2, or infiltrated with Avr2 alone. Leaves were photographed 3 days post infiltration. The infiltrated sectors are outlined and the infiltrated compounds indicated. The HR is only triggered when Rcr3 and Avr2 can interact, whereas the HR is blocked when interaction of Rcr3 with Avr2 is prevented by preincubation of Rcr3 with E-64. Rcr3 produced in *P. pastoris* treated with the same compounds gave similar results (not shown).

Materials and Methods

Plasmid constructs.

Throughout this study Rcr3 from *Lycopersicon esculentum* ($Rcr3^{pim}$) was used. The plasmids pMWBin19:$Rcr3^{pim}$-His-HA and pMWBin19 for transient expression in *Nicotiana benthamiana* are as described by Krüger et al., (2002). Plasmids for expression in *Pichia pastoris* were generated as follows. Vector pPIC-9 (Invitrogen) was modified by inserting an adaptor,which encodes a Histidine tag (6x His) and contains *Sma*I, *Apa*I and *Sac*II restriction sites, by ligating oligomers His-F and His-R (Table S1), into pPIG-9 using *Sna*BI restriction enzyme, resulting in vector pPIC-9His. To create His-FLAG-tagged Avr2, Avr2 was amplified from cDNA using primers Avr2-N and Avr2-C (Table S1) and cloned into pPIC-9His using *Sma*I and *Eco*RI restriction sites, resulting in plasmid pPIC-9His:FLAG-Avr2. The same strategy was followed to produce plasmids pPIC-9His:FLAG-Avr4 and pPIC-9His:FLAG-Ecp4, using primerpair Avr4-N and Avr4-C (Table S1) on *Avr4* cDNA, and primerpair Ecp4-N and Ecp4-C (Table S1) on *Ecp4* cDNA, respectively. To generate Rcr3-His-HA-tagged versions of Rcr3, Rcr3 was amplified from pMWBin19:Rcr3-His-HA (Krüger, et al. 2002) using primers Rcr3-N-Pro and Rcr3-Not-C (Table S1) and cloned into pPIC-9His using *Xho*I and *Not*I restriction sites, resulting in pPIC-9:Rcr3-His-HA.

Plant material

Nicotiana benthamiana and tomato (*Lycopersicon esculentum*) lines MoneyMaker (MM) Cf0, Cf2 and Cf2/*rcr3-3* were grown under standard greenhouse conditions with supplemental light (16hrs/day). Agroinfiltration was performed as described by Krüger, et al., (2002). Intercellular fluid (IF) was isolated by vacuum-infiltrating leaves with 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0, as described previously (de Wit and Spikman, 1982).

Protein expression

Pichia pastoris strain GS115 (Invitrogen) was transformed with *Sal*I-linearised pPIC-9His-derived plasmids (described above). Transformants were grown in BMMY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 1% methanol, 100mM K H_2PO_4, pH 6.0) daily supplemented with methanol to a final concentration of 1% (v/v). After removing cells by centrifugation, proteins in the culture supernatant (CS) were separated on Tricine SDS-PAGE (Schägger and Von Jagow, 1987), and stained with Coomassie Brilliant Blue or analyzed on Western blots, using antibodies against the various tagged proteins. Transformants expressing significant amounts of the different proteins were selected for fermentation. Fermentation was performed as previously described (van den Burg, et al., 2001). CS containing His-FLAG-Avr2, His-FLAG-Avr4 or His-FLAG-Ecp4 was concentrated on a YM03 filter (Amicon Ultrafiltration cell, model 402), and dialysed (Mw cut–off 3kD) overnight at 4°C to 50

mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0. The tagged proteins were subsequently purified on a Ni-NTA Superflow column (Qiagen; 30430) according to the manufacturer's protocol. The eluate fractions containing pure protein were pooled and dialysed to milli-Q water. Protein concentrations were determined by the BCA protein assay (Pierce). Proteins were tested for their HR-inducing activity by injection into leaves of tomato plants carrying the corresponding *Cf* genes. Production of Rcr3-His-HA by *P. pastoris* was checked by SDS-PAGE, Western blotting with α-HA-HRP, and protease activity profiling DCG-04 (see below).

Protease activity profiling

Protease activity profiling with DCG-04 was performed as previously described (*S4*, van der Hoorn, et al., 2004). Briefly, IF of leaves of *N. benthamiana* or CS of *P. pastoris*, was diluted 10 fold in DCG-04 assay buffer (50 mM NaAc, 10 mM L-cysteine, pH 5.0) to a final volume of 500 µl, DCG-04 (220 nM final concentration) was added and the reaction mixture was incubated at room temperature for 5 hrs. Proteins were precipitated by adding 1 ml of ice-cold acetone, washed with 70% (v/v) acetone and subsequently dissolved in 500 µl TBS buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.5). Biotinylated Rcr3-His-HA protein was affinity-purified on magnetic streptavidin beads (Promega) or on magnetic Ni-NTA beads (Qiagen) by incubating for 16 hrs at 4°C. Proteins were eluted from the beads by boiling in SDS sample buffer and analysed by SDS-PAGE and Western blotting. In inhibition studies of Rcr3 activity, the cysteine protease inhibitor E-64 and His-FLAG-Avr2 were tested for their ability to compete with DCG-04 (200 nM final concentration) for binding to Rcr3 at final concentrations of 35 nM, 70 nM, 140nM, 280 nM, 560 nM, 1120, nM and 2240 nM, respectively. The results are shown in Figure S1. From this Figure it is clear that DCG-04 can be fully displaced from Rcr3 by 1120 nM E-64 and 140 nM His-FLAG-Avr2, respectively.

Co-immunoprecipitation (Co-IP)

50 µl IF from *N. benthamiana* with or without Rcr3-His-HA was added to 450 µl Co-IP buffer (50 mM NaAc, 150 mM NaCl, 10 mM L-cysteine, pH 5.0) and mixed with either His-FLAG-Avr2 or His-FLAG-Avr4 (2,4 µM final concentration). To block the active site, Rcr3 was pre-incubated with E-64 (40 µM final concentration) for 20 min at room temperature before adding His-FLAG-Avr2. Samples were incubated overnight with 20 µl pre-equilibrated EZview α-FLAG M2 agarose beads (Sigma) at 4°C on a rotary shaker (15 rpm). The resin was subsequently collected by centrifugation at 3,000 *g* and washed in 1 ml Co-IP buffer. The bound proteins were eluted by Co-IP buffer containing 300 ng 3*FLAG peptide/µl (Sigma) and separated by SDS-PAGE. When CS from *P. pastoris* was used, NaCl was omitted from the Co-IP buffer and CS containing His-FLAG-Ecp4 was used as a negative control.

Induction of HR.

To 500 µl of IF from *N. benthamiana* or CS of *P. pastoris* expressing Rcr3-His-HA, 150 µl of DMSO or 150 µl of E-64 (2.3mM) in DMSO was added, and the mixtures were incubated for 5 hrs at room temperature. Subsequently, the samples were dialysed (Mw cut-off 3.5 kD) to milli-Q water and divided into two aliquots of 300 µl. To one aliquot 6 µl of Milli-Q water was added and to the other 6µl of His-FLAG-Avr2 (4.7µM). This resulted in four different samples: Rcr3, Rcr3+Avr2, Rcr3+E-64 or Rcr3+E-64+Avr2. In addition, samples of *P. pastoris*-produced His-FLAG-Avr2 or IF isolated from MM-Cf0 plants inoculated with race 5 of *C. fulvum* (containing Avr2) were used as controls. Each sample was injected in leaflets of *Cf-2/rcr3-3* tomato plants and HR was scored at three days after injection.

2. Supplementary Tables.

Table S1. Oligonucleotide primers.

Code	Sequence	Comment
His-F	GTACATCATCATCATCATCAT**CCCGGGCCCGCGG****	Forward adaptor His tag and sites for *Sma*I, *Apa*I and *Sac*II.
His-R	**CCGCGGGCCCGGG**ATGATGATGATGATGATGTAC	Reverse adaptor His tag and sites for *Sma*I, *Apa*I and *Sac*II.
Avr2-N	GACTACAAGGACGACGATGACAAGGCCAAAAAACTACCTGGCTG	FLAG-tag at 5' end of *Avr*2.
Avr2-C	CGC**GAATTC**TACGTATCATCAACCGCAAAGACCAAAACAG	*Eco*RI site at 3'end of *Avr2*.
Avr4-N	GACTACAAGGACGACGATGACAAGAAGGCCCCCAAAACTCAACC	FLAG-tag at 5'end of *Avr*4.
Avr4-C	CGC**GAATTC**TACGTATCATTGCGGCGTCTTTACCGGACACG	*Eco*RI site at 3'end of *Avr*4.
Ecp4-N	GACTACAAGGACGACGATGACAAGGACCCTTCCTTCCGCTTCAG	FLAG-tag at 5'end of *Ecp*4.
Ecp4-C	CGC**GAATTC**TACGTATCATTACGGGCAAGTGACCTG	*Eco*RI site at 3'end of *Ecp*4.
Rcr3-N-Pro	CCG**CTCGAG**AAAAGAGAGGCTGAAGCTGGAAGCCAGCCAAAAC TGTCC	*Xho*I site at 5'end of *Rcr*3.
Rcr3-Not-C	TTTTCCTTTT**GCGGCCGCC**TATGCTATGTTTGGATAAGAAGACATC TTTGCG	*Not*I site at 3'end of *Rcr*3

*The sequences in bold correspond to restriction sites

Table S2. Antibodies used in this study.

Antibody	Source	Dilution	Comment
α-His	Roche	1:500	Rcr3 detection
α-HA-HRP	Roche	1:1,000	Rcr3 detection
α-FLAG-M2	Sigma	1:3,000	Avr2 and Avr4 detection
α-FLAG-M2- biotinylated.	Sigma	1:1,000	Avr2 and Avr4 detection
Streptavidin-HRP	Sigma	1:3,000	Detection of biotinylated proteins
α-Mouse-HRP	Sigma	1:5,000	Secondary antibody for α-His
α-Rcr3 [a]	Eurogentec	1:2,000	Rcr3 detection

[a] α-Rcr3 was raised against peptide sequence 207-221 of $Rcr3^{pim}$ by Eurogentec (Belgium) following standard procedures.

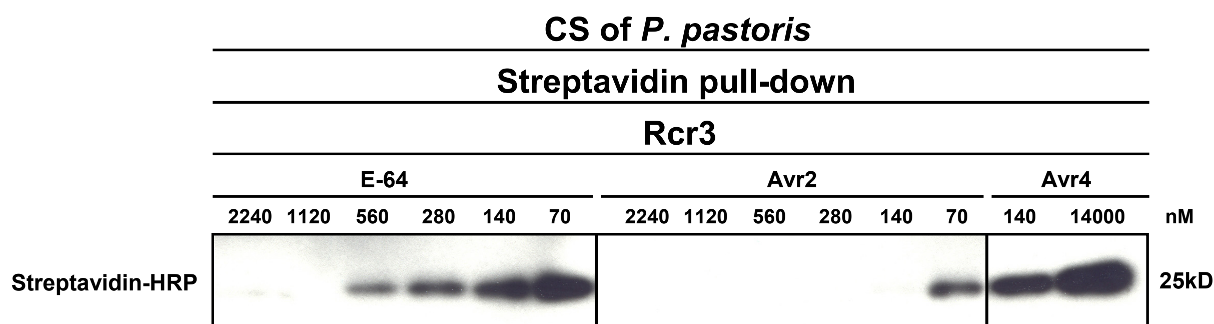

Figure S1. Abilities of Avr2 and E-64 to compete with DCG-04 for the active site of Rcr3-His-HA produced in *Pichia pastoris.*

Culture supernatant (CS) was isolated from a *P. pastoris* culture expressing Rcr3-His-HA (Rcr3) and was diluted 10-fold in protease assay buffer (endvolume 500 µl). The protease activity profiling was performed with DCG-04 at a constant concentration of 220 nM, in the presence of varying concentrations of the protease inhibitors E-64 or Avr2 (70 nM, 140 nM, 280 nM, 560 nM, 1120 nM and 2240 nM, respectively). As a negative control Avr4 was used in concentrations 140 nM and 14000 nM, respectively. Subsequently, biotinylated Rcr3 was pulled down by streptavidin beads, electrophoresed on an SDS gel and immuno-detected by streptavidin-HRP.

Note that eight times less Avr2 than E-64 is required to displace DCG-04 from Rcr3 completely.

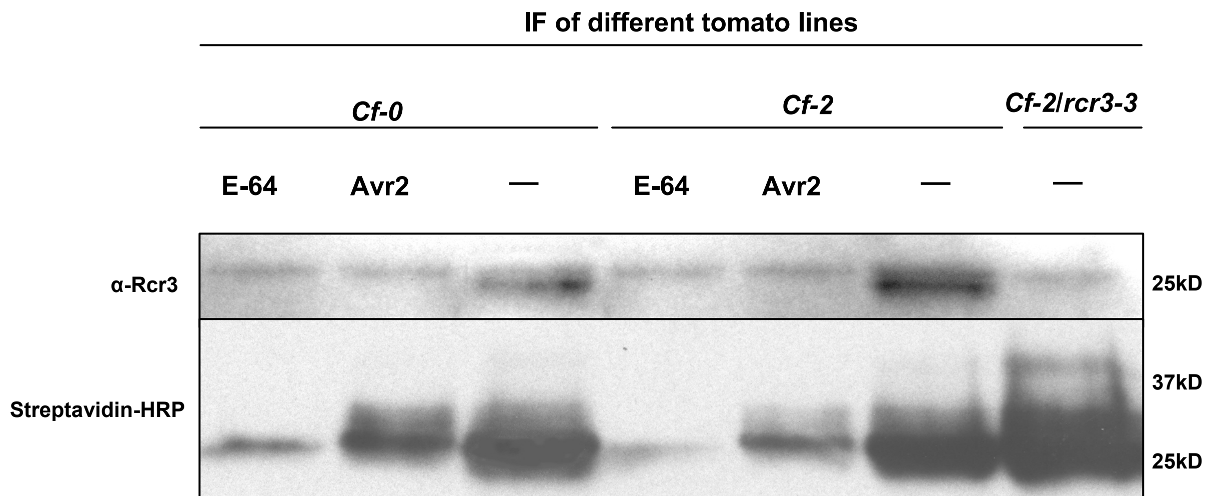

Figure S2. Activity profiling of native cysteine proteases present in IF of different tomato lines.

Six ml IF from *Cf-0, Cf-2* and *Cf-2/rcr3-3* tomato lines was subjected to protease activity profiling with DCG-04 (2.2 μM final concentration) in the absence of cysteine protease inhibitor (-), or in the presence of E-64 (28.6 μM final concentration) or Avr2 (6,9 μM final concentration). Subsequently biotinylated cysteine proteases were selected with streptavidin beads, electrophoresed on SDS gel, blotted onto nitrocellulose filters and probed with Rcr3-specific antibody (α-Rcr3) (upper panel). Note that Rcr3 is present in IF of *Cf-0* and *Cf-2* plants and its biotinylation is inhibited by both E-64 and Avr2. Rcr3 is absent in *Cf-2/rcr3-3* tomato. The lower protein band is Rcr3, the upper band recognized by α-Rcr3 is not a cysteine protease but likely a contamination with apoplastic acidic chitinase which cross-reacts with α-Rcr3 (results not shown). The western blot presented in the upper panel was stripped and re-probed with streptavidin-HRP (lower panel). Note that many cysteine proteases are labeled with biotin by DCG-04 in absence of cysteine protease inhibitor (-), whereas biotinylation is strongly inhibited in the presence of E-64 or Avr2.

CHAPTER 3

The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense

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Running title: Avr2 inhibits basal plant defense
Keywords: effector, Rcr3, Pip1, Cf-2, *Verticillium*, *Botrytis*, protease activity profiling, virulence target

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Dr. Ir. Bart P.H.J. Thomma (bart.thomma@wur.nl).

Synopsis:
The fungus *Cladosporium fulvum* secretes the effector protein Avr2 during infection of tomato. This work shows that Avr2 interacts with several extracellular cysteine proteases in tomato and Arabidopsis. *Avr2*-expression causes enhanced susceptibility towards several pathogens in both plant species and, with RNAi-mediated gene silencing in *C. fulvum*, we show that Avr2 is a genuine virulence factor.

Plant Cell, 20 (7), 1948-1963

ABSTRACT

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold of tomato. During growth in the apoplast, the fungus establishes disease by secreting effector proteins, ten of which have been characterized. We have previously shown that the Avr2 effector interacts with the apoplastic tomato cysteine protease Rcr3, which is required for Cf-2-mediated immunity. We now show that Avr2 is a genuine virulence factor of *C. fulvum*. Heterologous expression of *Avr2* in *Arabidopsis thaliana* causes enhanced susceptibility towards extracellular fungal pathogens including *Botrytis cinerea* and *Verticillium dahliae*, and microarray analysis showed that *Avr2* expression triggers a global transcriptome reflecting pathogen challenge. Cysteine protease activity profiling showed that Avr2 inhibits multiple extracellular Arabidopsis cysteine proteases. In tomato, *Avr2* expression caused enhanced susceptibility towards Avr2-defective *C. fulvum* strains, and also towards *B. cinerea* and *V. dahliae*. Cysteine protease activity profiling in tomato revealed that, in this plant also, Avr2 inhibits multiple extracellular cysteine proteases, including Rcr3 and its close relative Pip1. Finally, silencing of *Avr2* significantly compromised *C. fulvum* virulence on tomato. We conclude that Avr2 is a genuine virulence factor of *C. fulvum* that inhibits several cysteine proteases required for plant basal defense.

INTRODUCTION

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold of tomato (*Solanum esculentum*) (Joosten and de Wit, 1999; Thomma et al., 2005). Similar to other plant pathogenic Mycosphaerellaceae, host colonization is characterized by strict extracellular growth in the apoplastic space surrounding the leaf mesophyll without formation of haustoria (Bond 1938; Lazarovits and Higgins 1976; de Wit 1977; Thomma et al., 2005). Ten *C. fulvum* effector proteins secreted during host colonization have been characterized, the highest number for any filamentous plant pathogen studied so far (Thomma et al., 2005). Four of these effectors are race-specific avirulence proteins (Avr2, Avr4, Avr4E and Avr9), and six are extra-cellular proteins (Ecp1, Ecp2, Ecp4, Ecp5, Ecp6 and Ecp7), and all of the corresponding genes have been cloned (van Kan et al., 1991; van den Ackerveken et al., 1993; Joosten et al., 1994; Laugé et al., 2000; Luderer et al., 2002; Westerink et al., 2004; Bolton et al., 2008). Race-specific resistance against *C. fulvum* in tomato is governed in a gene-for-gene manner by dominant *C. fulvum* (*Cf*) resistance genes that mediate activation of a defense cascade, culminating in a hypersensitive response (HR) and host immunity (Joosten and de Wit, 1999; Rivas and Thomas, 2005). It has recently been demonstrated that the *C. fulvum* effector protein Avr4 (Joosten et al., 1994) contributes to full virulence by protecting fungal hyphae against hydrolysis by plant chitinases (van den Burg et al., 2006; van Esse et al., 2007). In addition, a putative intrinsic function was assigned to the Avr2 effector. In the incompatible interaction, Avr2 was shown to physically interact with, and inhibit, the extracellular papain-like cysteine protease Rcr3 (required for *C. fulvum* resistance; Krüger et al., 2002) which, in resistant tomato varieties, is guarded by the extracellular membrane-anchored resistance protein Cf-2 (Rooney et al., 2005). Interestingly, the Rcr3^{esc} variant that occurs in *S. esculentum* is a functional cysteine protease with a mutation outside the active centre of the enzyme that causes chronic necrosis in mature tomato plants carrying *Cf-2* (Krüger et al., 2002; Rooney et al., 2005). Thus, binding of Avr2 results in a conformational change of Rcr3 which is monitored by the Cf-2 protein, resulting in HR and resistance against *C. fulvum* isolates that produce wild-type Avr2 (Rooney et al., 2005).

Pathogens and their hosts use both proteases and protease inhibitors during their interactions to combat each other (van der Hoorn, 2008). Based on catalytic activity, the proteases are classified as aspartic, cysteine, metallo-, serine, and threonine proteases (Rawlings et al., 2006). These main classes have been further subdivided into clans and families based on evolutionary relationships. In the Arabidopsis genome, over 800 protease genes have been identified, of which approximately 140 are cysteine proteases including roughly 40 papain-like proteases (van der Hoorn, 2008). Several cysteine proteases have been demonstrated to play a role in programmed cell death-like responses, including pathogen-triggered HR (Beers et

al., 2000; Rojo et al., 2004; Suarez et al., 2004; Bozhkov et al., 2005; Kuroyanagi et al., 2005; Rooney et al., 2005; Hatsugai et al., 2006; Gilroy et al., 2007; Mur et al., 2007). Similar to Avr2, a number of secreted effector proteins with protease-inhibitory activity in tomato have been identified from the oomycete pathogen *Phytophthora infestans* (Tian et al., 2004; 2005; 2007). For example, a Kazal-like serine protease inhibitor targets the extracellular subtilisin-like protease P69B (Tian et al., 2004), and the cysteine protease inhibitor EPIC2, although structurally unrelated to Avr2, targets the Rcr3-like cysteine protease Pip1 (*Phytophthora*-inhibited protease; Tian et al., 2007). It is tempting to speculate that protease inhibitors such as *C. fulvum* Avr2 and *P. infestans* EPIC2 inactivate basal host defense by suppressing host protease activity, but so far it has not been demonstrated that these protease inhibitors genuinely contribute to pathogen virulence or that the targeted plant proteases are required for basal host defense.

In this study, we show that expression of *C. fulvum Avr2* in both Arabidopsis and tomato enhances susceptibility towards a number of fungal pathogens, including race 2 strains of *C. fulvum* that lack functional Avr2. Transcriptome analysis in Arabidopsis was employed to demonstrate that Avr2 does not merely disrupt normal host physiology, but triggers a global transcriptional reprogramming that reflects a typical host response to pathogen attack. Protease activity profiling was used to identify multiple host proteases in Arabidopsis and tomato that are inhibited by Avr2. Finally, we demonstrate with RNAi-mediated gene silencing that Avr2 contributes to *C. fulvum* virulence. Overall, the results of this study demonstrate that Avr2 is a genuine virulence factor of *C. fulvum* that inhibits a set of cysteine proteases that may be essential for basal host defense.

RESULTS

Heterologous Expression of *C. fulvum Avr2* in Arabidopsis Enhances Susceptibility Towards Distinct Fungal Pathogens

Basal defense against microbial pathogens is well characterized in the model plant Arabidopsis (Thomma et al., 2001). To assess whether any of the *C. fulvum* effector proteins targets conserved basal defense responses, transgenic Arabidopsis lines in the wild type Col-0 were generated that constitutively produce individual *C. fulvum* effector proteins. After segregation analyses, several independent homozygous single-integration lines were obtained for the *C. fulvum* effector genes *Avr2*, *Avr4*, *Avr4E*, *Avr9*, *Ecp2*, *Ecp4* or *Ecp5*. In a screen for attenuated basal defense, for each individual effector molecule three randomly chosen lines were challenged with the fungal pathogen *Botrytis cinerea* (Thomma et al., 1998; 1999a). In addition to increased susceptibility in the lines that express *Avr4* (van Esse et al., 2007), lines expressing *Avr2* also showed significantly enhanced grey mould disease, while transformants expressing any of the remaining effectors did not show clear alterations in disease susceptibility. As a result, the *Avr2*-expressing Arabidopsis lines were subjected to further analysis.

To select lines with the highest level of Avr2-production, total protein extracts were screened in western analyses using Avr2-specific polyclonal antibodies (van Esse et al., 2006). Three lines with the highest levels of Avr2 production were retained for further analyses (*At-Avr2-A* to *-C*, collectively called *At-Avr2* lines). When grown under standard greenhouse or climate chamber conditions, the *At-Avr2* lines did not show macroscopically visible phenotypic anomalies (Supplemental Figure 1A, B online). To confirm the presence of biologically active Avr2 in the apoplast of these transgenic lines, apoplastic fluid from *At-Avr2* lines was injected into leaves of a tomato *Cf-2* plant which resulted in a typical HR, while injection of apoplastic fluid from progenitor wild-type Col-0 plants showed only slight non-specific chlorosis (Supplemental Figure 1C online). Subsequently, the *At-Avr2* lines were challenged with the Arabidopsis fungal pathogens *Alternaria brassicicola*, *B. cinerea*, and *Plectosphaerella cucumerina*, the oomycete pathogens *Hyaloperonospora parasitica* and *Phytophthora brassicae* and the bacterium *Pseudomonas syringae* pv. *tomato* strain DC3000, using both progenitor Col-0 plants and *Avr9*-expressing Arabidopsis plants as controls. In these assays, *At-Avr2* lines inoculated with the necrotrophic fungal pathogens *B. cinerea* or *P. cucumerina* showed a clear enhancement of disease progression when compared to the inoculated control plants (Figure 1). On the *At-Avr2* lines necrotic lesions developed faster and grew larger compared to inoculated control plants on which lesions remained small (Figure 1; Supplemental Figure 2 online). Generally, disease progression did not result in sporulation of the pathogens on *At-Avr2* lines, although occasionally sporulation of *P. cucumerina* was observed on some of the inoculated leaves. Quantitative degrees of resistance are

typical for defense against necrotrophic pathogens (Thomma et al., 1998; 1999a; 1999b; Kliebenstein et al., 2005). Interestingly, inoculation with another necrotrophic fungus, *A. brassicicola*, did not result in a more susceptible phenotype, indicating that the enhanced susceptibility is pathogen dependent and not due to an overactive host defense. Likewise, inoculation with the bacterial pathogen *P. syringae* pv. *tomato* strain DC3000, or the oomycete pathogens *P. brassicae* and *H. parasitica* strains Waco9 (virulent) and Cala2 (avirulent), did not reveal increased susceptibility of *At-Avr2* lines. It has been shown previously that basal defense responses show differential effectivity towards different microbial pathogens (Thomma et al., 1998; 1999a; 1999b; 2000; 2001). Taken together, expression and extracellular targeting of Avr2 in Arabidopsis promotes the virulence of some, but not all, pathogens.

Avr2 **Expression in Arabidopsis Leads to Transcriptional Reprogramming Reflecting Defense Responses After Pathogen Challenge**

To investigate whether *Avr2* expression merely disturbs host physiology, or specifically interferes with basal host defences, transcriptional profiling in the absence of pathogen challenge was performed on four-week-old *At-Avr2-A* plants and progenitor Col-0 plants using Affymetrix ATH1 whole-genome arrays. In total, 880 genes were found to be significantly regulated (Bayesian t-testing, $P<0.05$) in response to *Avr2* expression. To gain insight into the underlying biological phenomena affected by constitutive *Avr2* expression, two complementary methods were applied. On the one hand, a scoring-based resampling method was applied to identify significantly overrepresented Gene Ontology (GO) classes (Lee et al., 2005). GO terms provide three structurally controlled vocabularies (ontologies) to describe genes and gene products in terms of their associated biological processes, their associated cellular components and their molecular and biochemical functions in a species-independent manner (Ashburner et al., 2000; Harris et al., 2004). As input, all t-test P values from the probe set comparisons across the respective conditions were used. To perform this method, the ErmineJ (Lee et al., 2005) software was used. This analysis revealed that genes participating in regulation of actin cytoskeleton reorganization, photosynthesis, and biosynthesis of nitrogenous compounds such as amino acids and glucosinolates were differentially regulated, in addition to pathways related to wounding, oxidative stress, and jasmonic acid/ethylene/salicylic acid-signaling (Supplementary Dataset 1 online). The cellular compartments associated with the differentially regulated gene products are involved in the secretory pathway and the exterior of the cell (apoplast), in addition to components that play a role in protein phosphorylation, reactive oxygen stress and proteasome function (Supplementary Dataset 1 online). This is also reflected by the molecular function of the products of the differentially regulated genes (Supplementary Dataset 1 online).

To further characterize the transcriptional response of *At-Avr2-A* plants, we employed Gene Set Enrichment Analysis (GSEA) which places gene products in a broader context covering biochemical, metabolic and signalling networks (Subramanian et al., 2005). This method is widely used to analyze human and murine transcriptome data, (van Baarlen et al., 2008). To perform GSEA for Arabidopsis, a database was constructed through transforming Arabidopsis KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/pathway.html) pathways information that represents current knowledge on molecular and biochemical networks. Furthermore, the database was supplemented with various expressed gene sets reported in literature. The resulting database was queried with the set of 880 Avr2-triggered differentially expressed Arabidopsis genes (Table 1). This showed that the obtained gene set was enriched for genes that are similarly found in Arabidopsis challenged with *P. syringae* or *Escherichia coli*, treated with bacterial effectors, or treated with pathogen phytotoxins such as *P. syringae* coronatine, *A. alternata* AAL toxin and *Fusarium oxysporum* Nep1 toxin (Table 1). Moreover, genes involved in the host secretory pathway were also over-represented (Table 1). Overall, it can be concluded from the global transcriptional profiling that *Avr2*-expression in Arabidopsis triggers a global transcription pattern that reflects pathogen challenge, suggesting that basal defense is affected rather than common physiological processes.

Identification of Arabidopsis Cysteine Proteases Targeted by Avr2

It has previously been demonstrated that Avr2 binds to, and inhibits, the tomato apoplastic cysteine protease Rcr3 (Rooney et al., 2005). To investigate whether Avr2 also inhibits Arabidopsis cysteine proteases, we applied protease activity profiling (van der Hoorn et al., 2004) on wild-type and *At-Avr2-A* plants. Total protein extracts from unchallenged soil-grown plants were treated with DCG-04, a biotinylated derivative of the irreversible cysteine protease inhibitor E-64 that reacts with the catalytic cysteine residue in an activity-dependent manner (Greenbaum et al., 2000) to biotinylate active cysteine proteases (van der Hoorn et al., 2004; Rooney et al., 2005). Subsequently, the biotinylated cysteine proteases were detected on western blots using streptavidin-coupled horseradish peroxidase (HRP), showing two major bands of biotinylated cysteine proteases in wild-type Col-0 plants migrating around 25 kDa and around 30 kDa. These bands can be fully competed by pretreatment with an excess of E-64 prior to labeling, indicating that DCG-04 specifically binds to cysteine proteases (Figure 2). Interestingly, treatment of total protein extracts from unchallenged soil-grown Col-0 plants with an excess of Avr2 likewise prevented subsequent labeling by DCG-04, demonstrating that Avr2 is also able to inhibit cysteine proteases in wild-type Arabidopsis plants (Figure 2). When compared to the progenitor Col-0 plants, a slightly different pattern of active cysteine proteases was observed in western blots of *Avr2*-expressing *At-Avr2-A* plants. In contrast to Col-0 plants, in *Avr2-A* plants, the 30 kDa band was more intense than the25 kDa band.

Pretreatment of the extracts with an excess of E-64 resulted in absence of active cysteine proteases, since no biotinylated signals were obtained on the blot (Figure 2), confirming that the signals were derived from active cysteine proteases. Interestingly, treatment of the sample with an excess of Avr2 did not prevent subsequent labeling by DCG-04, (Figure 2), demonstrating that the composition of the set of active cysteine proteases in *AtAvr2-A* plants is different from that in Col-0 plants and contains proteases that cannot be inhibited by Avr2.
To identify the cysteine proteases present in the extracts, biotinylated proteins present in DCG-04 labeled protein extracts were purified on streptavidin magnetic beads and subjected to LC/MS^2 analysis. In the progenitor Col-0 Arabidopsis plants, seven cysteine proteases could be identified, including aleurain, aleurain-like, cathepsin B, CPR1, RD21a, XCP1 and XCP2 proteases (Table 2). In the extract that was treated with an excess of E-64 prior to DCG-04 labeling, no proteases were identified at all. However, treatment of the Col-0 extract with an excess of Avr2 resulted in the detection of only cathepsin B (Table 2). This demonstrates that Avr2 is able to inhibit aleurain, aleurain-like, CPR1, RD21a, XCP1 and XCP2 because it can compete with DCG-04 for binding to the protease.
In *At-Avr2-A* plants, four cysteine proteases were detected: aleurain, aleurain-like, RD21a, and cathepsin B while CPR1, XCP1 and XCP2 were not detected (Table 2), demonstrating that constitutive *Avr2*-expression inactivates or represses CPR1, XCP1 and XCP2. Treatment of the extract of *At-Avr2-A* plants with an excess of E-64 prior to DCG-04 labeling resulted in the absence of detectable aleurain, aleurain-like, RD21a, and cathepsin B. However, treatment of the extract with an excess of Avr2 did not result in the absence of detectable aleurain, aleurain-like, RD21a, and cathepsin B, although the number of peptides derived from RD21A was significantly reduced. This suggests that the affinity of Avr2 for aleurain, aleurain-like and RD21a is lower than for CPR1, XCP1 and XCP2.
Overall, from the cysteine profiling experiments in Arabidopsis it can be concluded that the cysteine proteases in Arabidopsis can be divided into three classes based on their interaction with Avr2: proteases that cannot be inhibited by Avr2 (cathepsin B), proteases for which Avr2 has clear affinity (CPR1, XCP1 and XCP2), and proteases for which Avr2 has some (based on incidental treatment of Col-0 extracts) but rather low (based on the constitutive Avr2 presence in At-Avr2 plants) affinity (aleurain, aleurain-like and RD21a).

Production and Characterization of *Avr2*-Transgenic Tomato Lines

Using *A. tumefaciens*-mediated transformation, transgenic MoneyMaker-*Cf-0* tomato plants (MM-*Cf-0*) were generated for constitutive expression of *C. fulvum Avr2*. Two independent lines with a single copy insert of the transgene were retained for further analysis (MM-*Avr2-A* and MM-*Avr2-B*, collectively MM-*Avr2* lines). Similar to Arabidopsis, no macroscopically visible phenotypic anomalies were observed in

these lines when grown under standard greenhouse conditions (Supplemental Figure 3A online). It has previously been shown that tomato seeds expressing the *Cf-4* or *Cf-9* resistance gene in combination with the cognate *Avr* gene readily germinate, but develop a systemic HR within a few days post emergence of the hypocotyls and die (Cai et al., 2001; Hammond-Kosack et al., 1994; Thomas et al., 1997; Stulemeijer et al., 2007). Similarly, a cross between MM-*Avr2-A* and MM-*Cf-2* resulted in viable seeds that germinated at room temperature, but eventually all *Cf-2* x *Avr2* seedlings died, whereas seedlings from both parental lines retained normal germination and growth characteristics (Supplemental Figure 3C online). In addition, apoplastic fluids from MM-*Avr2* plants, but not those from the progenitor MM-*Cf-0* line, resulted in a clearly visible HR four days post injection into leaves of MM-*Cf-2* plants, confirming the presence of biologically active Avr2 in the apoplast of MM-*Avr2* lines (Supplemental Figure 3B online).

Heterologous Expression of *Avr2* in Tomato Promotes *C. fulvum* Colonization
Using the MM-*Avr2* lines, we determined whether *Avr2*-expression enhances the virulence of a wild-type strain of *C. fulvum* lacking functional Avr2. Four-week-old MM-*Avr2-A* plants and control MM-*Cf-0* plants were inoculated with conidia of a race 2 *C. fulvum* strain that lacks functional Avr2 and disease progression was monitored up to three weeks after inoculation. Visual inspection showed that *Avr2*-expressing plants were clearly more susceptible to this strain as colonization occurred faster than on MM-*Cf-0* plants (Figure 3). At 14 DPI, conidiophores of *C. fulvum* emerged from the *Avr2*-expressing plants, while conidiophores were not yet observed on MM-*Cf-0* leaves (Figure 3A). We have previously shown that *C. fulvum* biomass can be measured by real-time PCR determination of *C. fulvum* actin levels (van Esse et al., 2007; Bolton et al., 2008). The enhanced colonization of MM-*Avr2* plants was confirmed by real-time PCR at 10 and 14 DPI, showing that MM-*Avr2* plants accumulated more *C. fulvum* biomass faster than MM-*Cf-0* plants (Figure 3B). Similarly, enhanced colonization of MM-*Avr2-B* plants was observed when compared to control MM-*Cf-0* plants upon inoculation with race 2 *C. fulvum* (Supplemental Figure 4A online). Experiments with another natural *C. fulvum* strain that lacked a functional *Avr2* gene provided similar results (Supplemental Figure 4B online), strongly suggesting that Avr2 is a virulence factor of *C. fulvum*.

Silencing of *Avr2* in *C. fulvum* Compromises Virulence on Tomato
Gene silencing has been used reduce the expression of *C. fulvum* effector genes (van Esse et al., 2007; Bolton et al., 2008). To determine the role of Avr2, gene silencing was performed in a race 5 strain of *C. fulvum* (that contains the wild-type *Avr2* gene) using an inverted-repeat fragment of the *Avr2* gene driven by the constitutive *ToxA* promoter of the cereal pathogenic fungus *Pyrenophora tritici-repentis* (Ciuffetti et al., 1997). Several putative *Avr2* inverted-repeat (*Avr2*-IR)

transformants were obtained, three of which were used for further analysis. Growth of these transformants *in vitro* on potato dextrose agar was indistinguishable from that of the progenitor strain (Supplemental Figure 5 online). Since *C. fulvum* effector genes show no or low and variable expression when cultured *in vitro* (Thomma et al., 2006), four-week-old MM-*Cf-0* tomato plants were inoculated with the three independent *Avr2*-IR transformants to determine whether the introduction of the *Avr2*-IR resulted in silencing. The *in planta* expression levels of *Avr2* were determined relative to the constitutively expressed *C. fulvum* actin gene to calibrate for the amount of fungal biomass in the sample at 14 DPI using real-time reverse transcriptase PCR, showing a 60-70% reduction of *Avr2*-expression in each of the transformants when compared to the progenitor *C. fulvum* strain (Figure 4A). Nevertheless, these levels were still sufficient to trigger *Cf-2*-mediated resistance in MM-*Cf-2* plants since the *Avr2*-IR transformants were still avirulent, although the response to *Avr2*-IR transformants was less vigorous than to the progenitor *C. fulvum* strain (Supplemental Figure 6 online). Virulence assays on MM-*Cf-0* tomato plants showed that the *Avr2*-IR transformants were substantially compromised in their ability to colonize tomato leaves when compared to the progenitor *C. fulvum* strain, as they progressed slower and sporulated later (Figure 4B). This reduction in biomass by the *Avr2*-IR transformants as compared to the progenitor strain was confirmed by real-time PCR quantification of *C. fulvum* actin transcripts at 11 DPI (Figure 4C), demonstrating that Avr2 is a genuine virulence factor.

Heterologous Expression of *Avr2* in Tomato Enhances Disease Susceptibility

As *Avr2*-expressing Arabidopsis transgenic lines showed increased susceptibility towards various pathogens, we analyzed *Avr2*-expressing tomato lines for increased susceptibility towards *P. infestans*, *B. cinerea* and *Verticillium dahliae*. For *P. infestans*, no difference in susceptibility was observed between the MM-*Avr2* lines and the progenitor MM-*Cf-0* line. However, significantly more necrosis developed upon *B. cinerea* inoculation on MM-*Avr2* lines than on the progenitor MM-*Cf-0* line (Figure 5A), which correlated with enhanced fungal colonization as observed by microscopic analysis (Figure 5B). While on MM-*Cf-0* plants germinating *B. cinerea* conidia formed only short hyphae, hyphae on MM-*Avr2* plants were significantly longer and grew into the plant tissue (Figure 5B; Supplemental Figure 7 online). We subsequently tested the susceptibility of MM-*Avr2* plants towards the vascular pathogen *V. dahliae* (Fradin and Thomma, 2006). Also with this pathogen, enhanced disease development was observed on MM-*Avr2* plants as compared to the progenitor MM-*Cf-0* line. In this case, enhanced fungal colonization is reflected by stronger stunting, considerably reduced stem diameter, and stronger wilting symptoms in *Avr2*-transgenic lines (Figure 5C).

In our laboratory, we have also established a successful soil-based *V. dahliae*-infection assay for Arabidopsis (Fradin and Thomma, 2006). Like for tomato, we

found that *At-Avr2* plants were more susceptible to *V. dahliae* than progenitor Col-0 plants (Figure 5D).

Identification of Tomato Cysteine Proteases Targeted by Avr2

Apoplastic fluid obtained from a time course experiment of MM-*Cf-0* plants inoculated with a natural strain of *C. fulvum* lacking functional Avr2 (Boukema, 1981) were assessed for the presence of active cysteine proteases with biotinylated E-64. A western blot, using streptavidin coupled to horseradish peroxidase (HRP) for detection, demonstrated that inoculation of tomato with *C. fulvum* results in the induction of several active apoplastic cysteine proteases (Figure 6). Compared to 0 DPI, at five and seven DPI, more bands appeared while the intensities of the bands also increased, resulting in three major signals of 25, 30 and 37 kDa at seven DPI. The observed signals could fully be competed with an excess of E-64 prior to labeling, while they were largely competed by pre-treatment with Avr2 (Figure 6). This demonstrates that Avr2 is able to inactivate multiple cysteine proteases in tomato, as was also observed for Arabidopsis.

To identify the different tomato proteases, a large-scale labeling and purification experiment was performed. Cysteine proteases present in apoplastic fluids of non-inoculated MM-*Cf-0* plants were labeled with DCG-04, and biotinylated proteins were isolated using streptavidin beads and subsequently identified with LC/MS^2. Seven active cysteine proteases could be identified in the apoplast of non-inoculated MM-*Cf-0* leaves, including Rcr3, Pip1, TDI65, aleurain, glycinain, and two cathepsin B proteases (Table 3). Upon inoculation of MM-*Cf-0* plants with a natural strain of *C. fulvum* lacking a functional Avr2 gene, the same proteases were identified except glycinain, which disappeared upon infection (Table 3). This demonstrates that *C. fulvum* infection results in larger amounts of active cysteine proteases that are already present in non-inoculated tomato plants.

To determine potential targets of the cysteine protease inhibitor Avr2, apoplastic fluids of non-inoculated tomato leaves were treated with an excess Avr2 prior to labeling with DCG-04. In the extract that was treated with an excess of E-64 prior to DCG-04 labeling, no proteases were identified at all. However, after Avr2-treatment the Rcr3, Pip1 and glycinain proteases were no longer biotinylated by DCG-04 while TDI65, aleurain, and two cathepsin B proteases were still detected, showing that Avr2 has the highest affinity for Rcr3, Pip1 and glycinain (Table 3). The DCG-04 assay actually assesses the ability of Avr2 to block the binding of DCG-04 to active cysteine proteases. To directly assess the potential of Avr2 to interact with target cysteine proteases rather than assessing its competitive ability to block DCG-binding, Avr2 was labeled with biotin and used as bait to isolate and identify interacting apoplastic cysteine proteases with streptavidin beads in apoplastic fluids of non-inoculated tomato leaves. In this approach, the Rcr3, Pip1, TDI65 and aleurain proteases were found to bind to Avr2 (Table 3).

In addition to the non-inoculated and *C. fulvum*-inoculated MM-*Cf-0* plants, we performed protease activity profiling on apoplastic fluids from non-inoculated MM-*Avr2* plants. Compared to non-inoculated MM-*Cf-0* plants, five of the seven active cysteine proteases were also identified in MM-*Avr2* plants, the exceptions being Rcr3 and glycinain (Table 3). Interestingly, fewer peptides were identified for Pip1 and glycinain, suggesting that the relative amount of these proteases is reduced in the extract, which is also observed in extracts of MM-*Cf-0* plants upon pretreatment with Avr2 prior to DCG-04 labeling.

Overall, from the cysteine profiling experiments in tomato it can be concluded that the tomato cysteine proteases can be divided in three classes based on their interaction with Avr2: proteases that cannot be inhibited by Avr2 (cathepsin B), proteases for which Avr2 has clear affinity (Rcr3 and Pip1), and proteases for which Avr2 has lower affinity (TDI-65, aleurain, and glycinain). The inhibition of several extracellular host proteases by Avr2 is likely to cause the more susceptible phenotype. At present, biochemical evidence for the inhibition of protease activity by Avr2 only exists for Rcr3 (Rooney et al., 2005). However, *Cf-2/rcr3-3* mutants that lack Rcr3 due to a premature translational stop codon in the *Rcr3* gene (Krüger et al., 2002) did not show enhanced susceptibility towards race 2 *C. fulvum* strains that lack functional Avr2 when compared to *Cf-2/Rcr3* plants (Supplemental Figure 8 online), suggesting that loss of Rcr3 function alone is not sufficient for the enhanced disease susceptibility. Therefore, it is likely that the simultaneous inhibition of several host proteases by Avr2 causes the observed enhanced disease susceptibility phenotypes in different pathosystems.

DISCUSSION

In resistant tomato plants, the protease-inhibitory activity of *C. fulvum* Avr2 that results in modulation of the papain-like cysteine protease Rcr3 is monitored by the Cf-2 protein, which results in *Cf-2*-mediated disease resistance (Rooney et al., 2005). Here, we show that Avr2 is a general cysteine protease inhibitor that targets additional host proteases, which makes it a genuine virulence factor for *C. fulvum* that is also able to enhance the virulence of several other fungal plant pathogens on both tomato and Arabidopsis.

C. fulvum Avr2 Targets The Host Proteolytic Machinery

In this study, cysteine protease activity profiling was performed using the biotinylated E-64 inhibitor of C1 class of cysteine proteases, DCG-04. The profiling assays in tomato and Arabidopsis resulted in the identification of several extracellular cysteine proteases that interact with Avr2. Several proteases were identified in both hosts; cathepsin B and aleurain(-like), but also tomato TDI65, which is the homolog of Arabidopsis RD21A (Harrak et al., 2001). For the tomato proteases Rcr3 (Krüger et al., 2002) and Pip1 (Tian et al., 2007) no clear Arabidopsis homolog was identified. Likewise, for the Arabidopsis proteases XCP1, XCP2, (Zhao et al., 2000), and CPR1, no clear tomato homolog could be identified. However, XCP1 and XCP2 have been reported as xylem-specific C1 cysteine proteases (Zhao et al., 2000; Funk et al., 2002), and it should be noted that the Arabidopsis protease activity profiling was performed on whole plant extracts while the tomato profiling was performed on apopastic fluids. Thus, we are uncertain whether all identified Arabidopsis proteases indeed are present in the leaf apoplast.
To identify potential targets of Avr2 among the C1 proteases that irreversibly bind to E-64, the tomato and Arabidopsis extracts were treated with an excess of Avr2 prior to profiling with DCG-04. In both tomato and Arabidopsis extracts, Avr2 treatment prevented binding of DCG-04 to several proteases including tomato Rcr3, Pip1 and glycinain, and all Arabidopsis proteases except the cathepsin B and cathepsin B-like proteases (Tables 2 and 3). The ability to prevent DCG-04 binding to these proteases demonstrates the ability of Avr2 to interact with these targets. To further characterize Avr2 targets in tomato, biotinylated Avr2 was used to fish for targets in apoplastic fluids. Using this strategy, Rcr3, Pip1, TDI65 and the aleurain protease were identified. It is conceivable that the kinetics of the interaction in which Avr2 is used to inactivate proteases and thus block DCG-04 binding may be different from the kinetics in the interaction in which Avr2 is used as a bait to fish for targets. It is currently not possible to predict whether the kinetics of the different approaches used here are biologically meaningful or not. Nevertheless, these results confirm that Avr2 has the potential to target multiple host cysteine proteases of the C1 class, and that so far no additional targets could be found in the tomato apoplast fluids.

The finding that Avr2 pretreatment did not prevent binding of DCG-04 to TDI65 and aleurain, although these proteases were identified when fishing with biotinylated Avr2, suggests that Avr2 interacts reversibly with these two proteases. Furthermore, it should be noted that due to the limitations of the current LC/MS^2 technology our assay detected only qualitative but not quantitative differences between samples, so even a large reduction in binding of DCG-04 to TDI65 and aleurain may remain unnoticed. Clearly, in both tomato and Arabidopsis extracts, Avr2 treatment did not prevent binding of DCG-04 to cathepsin B proteases, suggesting that Avr2 has no affinity for these proteases (Tables 2 and 3). Overall, our assays demonstrate that *C. fulvum* Avr2 targets several apoplastic papain-like cysteine proteases of the host proteolytic machinery. Interestingly, our data furthermore show that, while *C. fulvum* Avr2 targets multiple host proteases, the tomato Cf-2 protein guards only Rcr3. This has also been observed for the *P. syringae* effectors AvrB, AvrRpm1 and AvrRpt2 that all target multiple host proteins of which only the basal defense regulator RIN4 is guarded by the cognate R proteins (Belkhadir et al., 2004; Lim and Kunkel, 2004; Chisholm et al., 2005). It was recently shown that treatment of tomato plants with the salicylic acid analog BTH induces the accumulation of Pip1 and Rcr3 (Shabab et al., 2008). Similar to our observations, it was shown in a competition experiment that, after Avr2-treatment, the Rcr3 and Pip1 proteases were no longer biotinylated by DCG-04, while the aleurain and cathepsin B proteases were still detected, thus confirming our finding that Avr2 has multiple targets in tomato (Shabab et al., 2008).

Host Proteases Are Essential For Basal Defense

Pathogens as well as their hosts use proteolytic machineries to modulate the outcome of their interaction. On the one hand, several bacterial effectors have been identified that possess protease activity to degrade or modify host components (Hotson and Mudgett, 2004). For instance, *P. syringae* AvrPphB targets the host protein kinase PBS1 (Shao et al., 2003), and AvrRpt2 cleaves the Arabidopsis basal defense regulator RIN4 (Kim et al., 2005a; 2005b). Consistent with the guard hypothesis, in both cases the plant has developed guards to monitor this effector-mediated degradation (RPS5 and RPS2, respectively) that subsequently activate effector-triggered immunity. On the other hand, host proteases are important for defense against pathogens (van der Hoorn, 2008). For example, the Arabidopsis aspartic protease CDR1 is proposed to mediate a peptide signal system involved in the activation of inducible resistance against *P. syringae* (Xia et al., 2004), while the vacuolar cysteine protease RD19 is required for RRS1-R resistance that is triggered by the PopP2 effector of *Ralstonia solanacearum* (Bernoux et al., 2008). Both RD19 and RRS1-R are targeted by PopP2 and are translocated to the nucleus where effector-triggered immunity is activated. Furthermore, several plant proteases have been implicated in the HR (D'Silva et al., 1998; Solomon et al., 1999; Coffeen and Wolpert, 2004; Rojo et al., 2004; Woltering, 2004; Chichkova et al., 2004; Gilroy et

al., 2007). It is therefore not surprising that pathogens utilize protease inhibitors during infection to target host proteases. Several secreted effector proteins from the oomycete pathogen *P. infestans* have been identified that display protease-inhibitory activity (Tian et al., 2004; 2005; 2007). The Kazal-like serine protease inhibitor directly interacts with the extracellular subtilisin-like protease PR-protein P69B (Tian et al., 2004), while the cysteine protease inhibitor EPIC2 interacts with the cysteine protease Pip1 (Tian et al., 2007). However, while diverse roles of plant proteases in disease signaling have been established, a role as genuine defense molecule has so far not been demonstrated.

In this study, apoplastic delivery of Avr2 in Arabidopsis, a non-host for *C. fulvum*, and in tomato resulted in enhanced susceptibility towards several fungal pathogens. In addition to the biotroph *C. fulvum*, these include the necrotrophic pathogens *B. cinerea* and *P. cucumerina*, and the vascular pathogen *V. dahliae*. However, no enhanced susceptibility towards the avirulent fungal pathogen *A. brassisicola* and the bacterial pathogen *P. syringae* was observed in *Avr2*-expressing Arabidopsis. Likewise, disease development by the haustorial pathogens *H. parasitica*, *P. brassicae* and *P. infestans* remained unaltered. Taken together, these results demonstrate that Avr2 expression compromises basal defense against pathogens that may be designated as extracellularly growing (non-haustorial) virulent fungi. This likely reflects that pathogens that do not utilize haustoria or mechanisms for host cytoplasmic delivery of effector proteins such as type III secretion or RxLR host targeting motifs, are more sensitive to apoplastic defenses.

Compromising specific defense mechanisms by Avr2, rather than merely disturbing host physiology, is not only supported by the disease susceptibility towards specific pathogens, but is further substantiated by transcriptional profiling of *Avr2*-expressing Arabidopsis plants in the absence of pathogen challenge. GSEA and ErmineJ analyses (Lee et al., 2005; Subramanian et al., 2005) were used to characterize the transcriptional response of Arabidopsis upon *Avr2*-expression as a typical plant response to pathogens or pathogen-derived components. Both types of analyses are unbiased because no gene selection step is used as all expressed genes are included, and a score is computed based on all genes in a particular GO term or gene set. Genes involved in the regulation of actin cytoskeleton reorganization and typical responses to wounding, oxidative stress, jasmonic acid, ethylene and salicylic acid (Supplementary Dataset 1 online) were overrepresented in the expression profiles. Furthermore genes associated with the secretory pathway and the exterior of the cell (apoplast) were also overrepresented.

Identification of Intrinsic Roles of Filamentous Pathogen Effectors

A role for secreted effectors in pathogen virulence has been demonstrated for only a few filamentous pathogens. Three *in planta* secreted *C. fulvum* proteins, Ecp1, Ecp2 and Ecp6, have been implicated in full virulence of the pathogen (Laugé et al., 1997;

van Esse et al., 2007; Bolton et al. 2008). Similarly, two avirulence proteins from the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* and the *SIX1* avirulence protein from *Fusarium oxysporum* f. sp. *lycopersici* were shown to increase fungal infectivity on the respective hosts (Rep et al., 2005; Ridout et al., 2006). The secreted effector proteins ATR1 and ATR13 from the oomycete Arabidopsis pathogen *H. parasitica* were also shown to contribute to pathogen virulence when delivered to the host by *P. syringae* (Sohn et al., 2007). However, in all these cases, the mechanism by which these effectors contribute to virulence is not yet understood. In a recent study to investigate the mechanism of action of a microbial effector, it was demonstrated that the *C. fulvum* effector protein Avr4 is a counter-defense factor that protects fungal cell walls against hydrolysis by plant chitinases through chitin-binding activity, and thus contributes to fungal virulence (van Esse et al., 2007). We have now shown that the *C. fulvum* effector protein Avr2 contributes to fungal virulence by targeting host proteases that are crucial for basal defense since *Avr2*-expressing tomato is more susceptible towards natural race 2 *C. fulvum* strains and *Avr2*-silencing in a race 5 strain of *C. fulvum* clearly affected fungal aggressiveness.

Importantly, our results demonstrate that heterologous expression of secreted pathogen effectors *in planta* may successfully be used to uncover the intrinsic biological functions of these molecules. Moreover, depending on the nature of the effector target, the plant species used may even be a non-host of the pathogen from which the effector is derived. We have recently used heterologous expression in Arabidopsis and tomato to show that the *C. fulvum* effector Avr4 is a genuine virulence factor (van Esse et al., 2007) and in the present study, we used a similar approach for *Avr2*. Several virulence targets of Avr2 were identified both in Arabidopsis and in tomato, while increased susceptibility towards some of the same pathogens was demonstrated. This not only suggests that basal defense responses in different plant species are highly conserved, but also that effector targets of different pathogens with diverse hosts may be orthologs (van Baarlen et al., 2007).

METHODS

All experiments have been performed a minimum of three times yielding similar results.

Cultivation of Micro-organisms and Plants

C. fulvum and *V. dahliae* were cultured at room temperature on half-strength potato dextrose agar (Oxoid, Basingstoke, England) supplemented with 7 g/l technical agar (Oxoid, Basingstoke, England). *B. cinerea* (Brouwer et al., 2003) and *P. cucumerina* (Thomma et al., 2000) were cultured at room temperature on malt extract agar (Oxoid, Basingstoke, England). *P. brassicae* isolate CBS686.95 was grown on V8 juice (Campbell Soups) agar (Erwin and Ribeiro, 1996) in the dark at 18°C. *P. syringae* pv. *tomato* DC3000 was cultured on King's B medium containing 200 µg/ml rifampicin.

Cf2/rcr3-3 tomato was described previously (Krüger et al., 2002). All tomato plants were grown in soil under standard greenhouse conditions: 21°C/19°C during the 16 h day/8 h night period, 70% relative humidity (RH) and 100 W/m^2 supplemental light when the intensity dropped below 150 W/m^2. Arabidopsis plants were grown in soil under similar greenhouse conditions with 21°C/18°C during the 16 h day/8 h night, 60% RH and 100 W/m^2 supplemental light when the intensity dropped below 150 W/m^2.

Plant Transformations

Transgenic Arabidopsis (*At-Avr2-A* to *-C*, collectively called *At-Avr2* lines) and tomato (MM-*Avr2-A* and MM-*Avr2-B*, collectively MM-*Avr2* lines) expressing *C. fulvum Avr2* were generated in this study. Transgenic Arabidopsis expressing Avr4 and Avr9 have been described previously (van Esse et al., 2007). For *in planta* production of *C. fulvum* effectors, the sequence encoding each of the mature proteins was amplified (Supplemental Table 1 online) and ligated into the binary pGREEN vector that contained the CaMV 35S promoter for constitutive expression (Hellens et al., 2000) in frame with the sequence encoding the tobacco PR1a signal peptide for apoplastic targeting. This vector was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and transformants were selected on LB medium supplemented with 50 µg/ml kanamycin and 25 µg/ml rifampicin. Subsequently, Arabidopsis transformants were generated using 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 T_3 and T_4 lines were used for inoculations.

Tomato transformations were performed using a modified protocol of Cortina and Culiáñez-Macià (2004). Seeds of the tomato cultivar MoneyMaker (MM-Cf-0) were surface-sterilized (by incubation for 1 minute in 70% EtOH, 25 minutes in 10% commercial bleach and rinsing three times in sterilized water), sown on MS agar

supplemented with sucrose (30 g/l), incubated in the dark in a growth chamber at 25°C for two days and subsequently exposed to light. After approximately 10 days, cotyledons were harvested, cut in two, and placed upside down in Petri dishes containing pre-cultivation medium (MS agar supplemented with 30 g/l sucrose, 2 mg/l NAA, 1 mg/l BAP, pH 5.8), after which the explants were covered with sterile filter paper imbibed with 2 ml of co-cultivation medium (MS medium supplemented with 30 g/l sucrose, 2 g/l caseine hydrolysate (Duchefa), 1 g/l 2,4 D, 0.5 mg/l kinetine (dissolved in 1 M NaOH), pH 6.5) and incubated in the dark for 24 hours. Transgenic *A. tumefaciens* carrying the construct of interest was grown in LB medium containing 200 µM acetosyringone to an OD_{600} of 0.6, and after harvesting, the bacteria were re-suspended in 75 ml of LB medium. Subsequently, the explants were incubated in the bacterial suspension for 5 to 10 minutes, dried on sterile filter paper, plated on pre-cultivation medium, and incubated in the dark for two days. The explants were then transferred to regeneration medium (MS agar supplemented with 10 g/l sucrose, 10 g/l glucose, 2 mg/l zeatin riboside, 0.4 mg/l thiamine-HCL, 0.02 mg/l IAA, 200 mg/l timentin (ticarcilline:potassium clavulanate [15:1], 100 mg/l kanamycin, 200 mg/l vancomycin, pH 5.8), incubated in the dark for five days, and transferred into light. The explants were transferred to fresh regeneration medium every two weeks. When calli appeared, they were transferred to shoot-inducing medium (MS agar supplemented with 10 g/l sucrose, 10 g/l glucose, 1 mg/l zeatin riboside, 0.02 mg/l IAA, 200 mg/l timentin, 100 mg/l kanamycin, 200 mg/l vancomycin, pH 5.8). Upon meristem development, the explants were transferred to root-inducing medium (MS agar supplemented with 10 g/l sucrose, 10 g/l glucose, 0.02 mg/l IAA, 200 mg/l timentin, 50 mg/l kanamycin, pH 5.8). Once roots developed, the plantlets were planted in soil and transferred to the greenhouse where they were grown under standard greenhouse conditions.
To verify apoplastic delivery of Avr2 in transgenic Arabidopsis and tomato plants, apoplastic fluid was isolated and injected into *Cf-2* tomato in order to obtain an HR according to de Wit and Spikman (1982).

Plant Inoculations
Inoculation of tomato with *C. fulvum* was performed as previously described (de Wit, 1977). To assess susceptibility of the *Avr2*-expressing tomato lines, the Avr2-deficient *C. fulvum* strains 2.4.5 (Boukema, 1981) and 2.5.9 (Laterrot, 1986) were used. Briefly, five-week-old soil-grown tomato plants were inoculated by spraying 5 ml of conidial suspension (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 we incubated at standard greenhouse conditions of 16 h/8h light/dark regime and 70% RH. Disease progression was monitored until 20 DPI.
Inoculation of tomato with *B. cinerea* (Brouwer et al., 2003) was performed as previously described (Díaz et al., 2002) with slight modifications. Briefly, a

suspension of 10^6 conidia/ml in Gamborg's B5 medium (Duchefa Biochemie bv, Haarlem, The Netherlands) supplemented with 10 mM Glc and 10 mM potassium phosphate (pH 6) was pre-incubated without shaking for 2 to 3 h at room temperature. Subsequently, five-week-old soil-grown tomato plants were inoculated by spraying 5 ml the inoculum onto the lower surface of the leaves. Plants were kept at 100 % RH for 48 h under a transparent plastic cover after which they we incubated at standard greenhouse conditions. For microscopic analysis, *B. cinerea* inoculated leaves were stained with lactophenol-trypan blue (Keogh et al., 1980) to visualize hyphal structures and death plant cells. Leaves were destained and mounted in a chloral hydrate solution (2.5 mg/ml). Microscopy was preformed with a Nikon 90i epifluorescence microscope equipped with a Nikon DS-5Mc digital imaging system and Nikon NIS-elements AR software 2.3 (Nikon Instruments inc, Melville, NY, U.S.A.).
P. infestans inoculations on tomato were performed on detached leaves as described for potato (Vleeshouwers et al., 1999).
For inoculation with *V. dahliae*, two-week-old soil-grown tomato plants were up-rooted and inoculated by dipping the roots for 2 minutes in a conidial suspension (10^6 conidia/ml) in water. After re-planting in soil, plants were incubated at standard greenhouse conditions of 16 h/8h light/dark regime and 70% RH. Disease progression was monitored until 20 DPI.
Inoculation of Arabidopsis plants with *B. cinerea, P. cucumerina*, *P. brassicae* and *P. syringae* were performed on four-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 12 g/l potato dextrose broth (Difco, Leeuwarden, The Netherlands) on each leaf. Inoculation with *P. cucumerina* was performed similarly, using an aqueous suspension containing 5×10^5 conidia/ml. For all pathogens, plants were incubated at 20°C, 100% RH and a 16 h/8h light/dark regime. Disease progression was scored at regular intervals and representative pictures were taken at four DPI. Inoculation with *P. brassicae* was performed by placing 5 mm-diameter plugs of a two-week-old *P. brassicae* agar plate culture onto Arabidopsis leaves. Subsequently, the plants were incubated at 16°C, 100 % RH and a 16 h/8h light/dark regime. Inoculation with *P. syringae* pv. *tomato* DC3000 was performed by spray inoculation of a bacterial suspension of 5×10^8 cfu/ml in 10 mM $MgCl_2$, 0.05% Silwet L-77 (Lehle Seeds, Round Rock, TX, USA) 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 h/8h light/dark regime. Disease progression was scored at four DPI.
For inoculation of Arabidopsis with *V. dahliae*, two-week-old soil-grown plants were up-rooted and inoculated by dipping the roots for 2 minutes in a conidial suspension (10^6 conidia/ml) in water. After re-planting in soil, plants were incubated at standard greenhouse conditions of 16 h/8h light/dark regime and 60% RH. Disease progression was monitored until 20 DPI.

Microarray Sample Preparation and Data Analyses

Samples used for microarray analyses were replicated three times in independent experiments and each replication consisted of 10 *At-Avr2-A* Arabidopsis plants and 10 Col-0 plants grown for four weeks under standard greenhouse conditions. Thus, in total 30 *At-Avr2-A* plants and 30 Col-0 plants were assayed. All above-ground tissues were harvested, pooled and flash-frozen in liquid nitrogen. For RNA extraction, the frozen leaves were ground using a spoon and approximately 100 mg of the crushed material was homogenized in 1 ml Trizol® reagent (Invitrogen, Carlsbad, CA, USA). After addition of 200 µl chlorophorm and phase separation, isopropanol was added to the aqueous phase, which was subsequently further purified with the NucleoSpin RNA plant kit (Macherey-Nagel GmbH, Düren, Germany). In this way, total RNA was obtained that was hybridized onto six individual ATH1 Affymetrix Arabidopsis whole-genome arrays from *At-Avr2-A* and Col-0 plants grown in three independent replications. Probe preparations and GeneChip hybridizations were carried out at ServiceXS (Leiden, The Netherlands).

Bioconductor packages (www.bioconductor.org; Gentleman et al., 2004) were used for analysing the scanned Affymetrix arrays. The Bioconductor packages were integrated in the automated on-line MADMAX pipeline (https://madmax.bioinformatics.nl). The arrays were normalised using quantile normalisation, and expression estimates were compiled using RMA applying the empirical Bayes approach (Wu et al., 2004). They were considered of sufficiently high quality if they showed less than 10% of specks in fitPLM model images, were not deviating in RNA degradation and density plots, were not significantly deviating in NUSE and RLE plots, and were within each other's range in boxplots. Differentially expressed probesets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularisation of standard errors (Smyth, 2004). P-values were corrected for multiple testing using a false discovery rate (FDR) method (Storey et al., 2003) that estimates type 1 (false positive) errors. For plants, an FDR < 0.05 cut-off is a suitable global value when arrays are of high quality (DeCook et al., 2006). Two complementary methods were applied to relate changes in gene expression to functional changes. One method is based on overrepresentation of Gene Ontology (GO) terms (Lee et al., 2005). Another approach, gene set enrichment analysis (GSEA), takes into account the broader context in which gene products function, namely in physically interacting networks, such as biochemical, metabolic or signal transduction routes (Subramanian et al., 2005). Both methods are unbiased, because no gene selection step is used. Moreover, since a score is computed based on all genes in a particular GO term or gene set, the signal-to-noise ratio is boosted allowing the detection of transcriptional programs that are distributed across an entire set of interacting genes yet are subtle at the level of individual genes. The higher FDR values reflect the biological variation

that consists of between-plant variation and variation resulting from multiple (three) independent plant rearing and harvesting dates.

Avr2 Inverted-Repeat Transformants of *C. fulvum.*

A fragment of the *Avr2*-coding sequence was amplified using cDNA from a compatible interaction between *C. fulvum* and tomato as template using the primer sequences as shown in Supplemental Table 1 online. Construction of the binary vector containing an inverted repeat fragment of the *Avr2* gene and *A. tumefaciens*-mediated transformation of a race 5 strain of *C. fulvum* was performed as described (Bolton et al., 2008).

Quantification of *Avr2* Expression Levels and *C. fulvum* Biomass.

The *Avr2*-IR transformants and the progenitor race 5 *C. fulvum* strain were inoculated onto five-week-old MM-*Cf-0* tomato plants as described above. In each of three independently repeated experiments, leaf material was harvested 0, 3, 7, 11 and 16 DPI, flash-frozen in liquid nitrogen and stored at -80°C until used for RNA analysis. Leaf samples consisted of three leaflets obtained from the 2nd, 3rd, and 4th compound leaves of two tomato plants. Total RNA was isolated from infected leaf material using the RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA was used for cDNA synthesis using an oligo(dT) primer (Supplemental Table 1 online) and the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was conducted with primers given in Supplemental Table 1 online, and using an ABI7300 PCR machine (Applied Biosystems, Foster City, CA, USA) in combination with the qPCR Core kit for SYBR® Green I (Eurogentec, Seraing, Belgium). Real-time PCR conditions were as follows: an initial 95°C denaturation step for 2 min followed by denaturation for 15s at 95°C and annealing/extension for 45s at 60°C for 40 cycles and analyzed on the 7300System SDS software (Applied Biosystems, Foster City, CA, USA). To check for contamination with genomic DNA, real-time PCR was also carried out on RNA without the addition of reverse transcriptase. Quantification of *C. fulvum* growth on MM-*Avr2* lines was performed similarly. Statistical analyses were performed in SPSS15.0 using one-way analysis of variance (ANOVA; $P<0.05$) followed by the LSD and Dunnett t (2-sided) post hoc multiple comparisons.

Identification of Plant Cysteine Proteases Targeted by Avr2

Protein extracts from Arabidopsis and tomato were prepared and subjected to protease activity profiling with DCG-04 (van der Hoorn et al., 2004). In the profiling assays, the cysteine protease inhibitors E-64 (110 µM final concentration) and His-FLAG-Avr2 (11 µM final concentration) were tested for their ability to compete with DCG-04 (220 nM final concentration) for binding to cysteine proteases.

For tomato, apoplastic fluid was isolated from MM-*Cf-0* tomato inoculated with the Avr2-deficient *C. fulvum* strain (Boukema, 1981) at 14 DPI as previously described (van Esse et al., 2006), and 9 ml of fluid was used for protease activity profiling. To each extract, 1 ml of DCG-04 assay buffer (500 mM NaAc, 100 mM L-cysteine, pH 5.0) with DCG-04 (2.20 µM final concentration) was added and incubated at room temperature for 5 hrs. Subsequently, proteins were precipitated by addition of 20 ml of ice-cold acetone, washed with 70% (v/v) acetone and subsequently dissolved in 1 ml TBS buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.5). The biotinylated cysteine proteases were bound to magnetic streptavidin beads (Promega, Madison, USA) by incubating for 16 hrs at 4°C. The beads were washed 3 times (50 mM Tris/HCl, 1.15 M NaCl and 1% Triton X100) and subsequently rinsed twice with 50 mM NH_4HCO_3 (pH 8.0). To reduce disulphide bridges, the beads were incubated with 50 mM dithiotreitol in 50 mM NH_4HCO_3 (pH 8.0) for 2 hrs at 56ºC, followed by alkylation of cysteine residues by incubation in 50 mM iodoacetamide in 50 mM NH_4HCO_3 (pH 8.0) for 2 hrs at 25 ºC in the dark. Finally, the immobilized cysteine proteases were subjected to trypsin digestion. To this end, a fresh stock of 20 µg trypsin (Promega, Madison, USA) in l00 µl 50 mM HAOc was prepared. Four µl of this stock solution was diluted 10-fold in 100 mM NH_4HCO_3 (pH 8.0), added to the beads, and incubated overnight at room temperature. Subsequently, another 4 µl of stock solution was added and incubated for 4 hrs at 37ºC. The supernatant containing tryptic digests was separated from the magnetic beads, and 22 µl of the suspension was subjected to LC/MS^2 analysis.
For Arabidopsis, isolation of cysteine proteases was performed as described previously (van der Hoorn et al., 2004), and reduction of disulphide bridges and tryptic digests were performed as described above for tomato.
To identify cysteine proteases that directly bind to Avr2, the above-described protease activity profiling assays were performed in which DCG-04 was replaced by biotinylated Avr2 (67 µM final concentration). Biotinylated Avr2 was produced by labeling of *Pichia pastoris*-produced Avr2 (Rooney et al., 2005) using the No-Weigh™ Premeasured NHS-PEO4-Biotin Microtubes (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.
The protein samples were analyzed with LC/MS^2 by injecting 18 or 20 µl of sample on a 0.10 x 32 mm Prontosil 300-3-C18H pre-concentration column (Bischoff, Leonberg, Germany) at a flow of 3 or 6 µl/min for 10 minutes. Peptides were eluted from the pre-concentration column onto a 0.10 x 200 mm Prontosil 300-3-C18H analytical column (Bischoff, Leonberg, Germany) with an acetonitril gradient at a flow of 0.5 µl/min. The gradient consisted of a 10 to 35% (v/v) acetonitril increase in water with 1 ml/l formic acid in 50 minutes. As a subsequent cleaning step, in 3 minutes the acetonitril concentration was increased to 80% (v/v) (with 20% water and 1 ml/l formic acid in both the acetonitril and the water).

Downstream of the analytical column, an electrospray potential of 1.8 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P875 Upchurch microT. Full scan positive mode MS spectra with 3 microscans (LCQ) or 1 microscan (LTQ) were measured between m/z 350 or 380 and 1400 on a LCQ classic or LTQ-Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA). The equipment was optimally tuned either by direct injection of 1 µM bradykinin or by injection of positive mode calibration mix at 0.5 µl/min via the electrospray device mentioned above. MS^2 scans of the three or four most abundant peaks in the MS scan were recorded in data dependent mode.

All MS^2 spectra were analyzed with Bioworks 3.2 or 3.3.1 software (Thermo Fisher Scientific, San Jose, CA, USA). A maximum of 3 differential modifications was set for oxidation of methionines and de-amidation of N and Q. Carboxamidomethylation of cysteines was set as a fixed modification. An *Arabidopsis thaliana* database (downloaded from the European Bioinformatics Institute website at http://www.ebi.ac.uk/integr8/) to which the following protein sequences were added: BSA (P02769, bovin serum albumin precursor), Trypsin (P00760, bovin), Trypsin (P00761, porcin), Keratin K2C1 (P04264, human) and Keratin K1CI (P35527, human) was used for peptide identifications. The peptide identifications obtained were filtered in Bioworks with the following filter criteria: $\Delta Cn > 0.08$, Xcorr > 2 for charge state 1+ , Xcorr > 1.5 for charge state 2+, Xcorr > 3.3 for charge state 3+ and Xcorr > 3.5 for charge state 4+ (Peng et al., 2003).

Accession Numbers

Sequence data from this article can be found in TIGR or GenBank/EMBL databases. The accession numbers can be found in Tables 2 and 3.

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Figure and figure legends

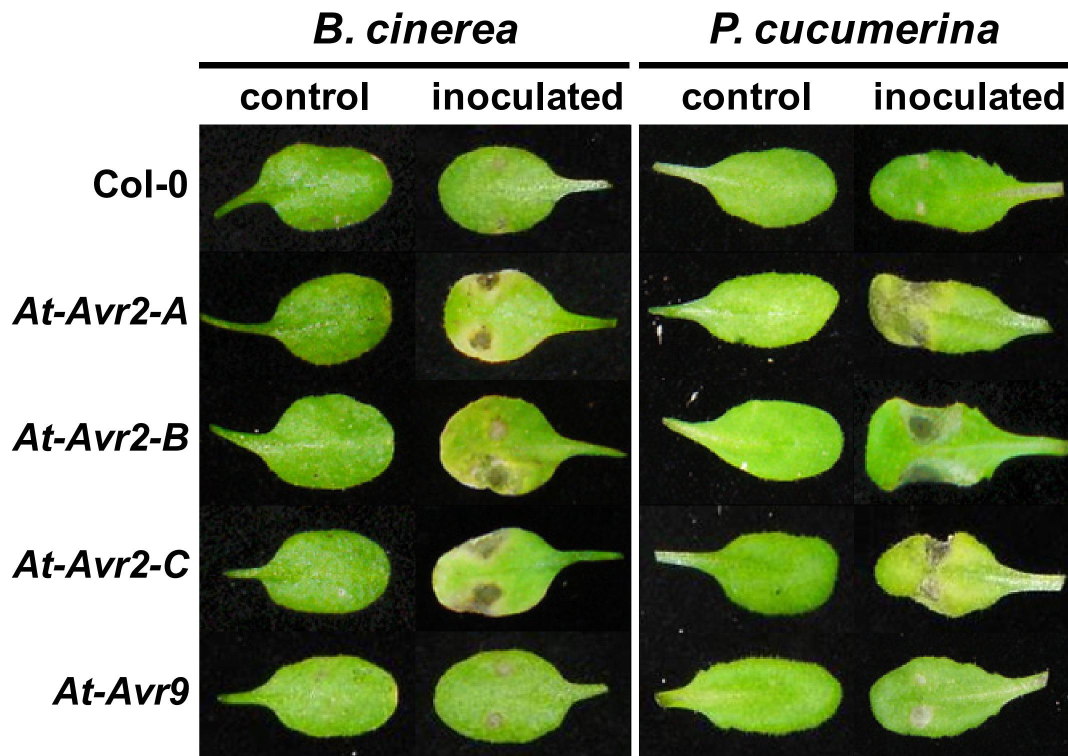


Figure 1. *Avr2*-Expressing Arabidopsis Is More Susceptible to the Fungal Pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*.

Typical symptoms caused by *B. cinerea* and *P. cucumerina* on four-week-old plants of three independent *Avr2*-expressing Arabidopsis lines (*At-Avr2-A* to *-C*) at four DPI. Typical symptoms on the progenitor Col-0 line and an *Avr9*-expressing transgenic line (*At-Avr9*) are shown as controls.

Figure 2. Avr2 Inhibits Cysteine Proteases in Arabidopsis.
Western blot of total protein extracts from Arabidopsis transformants expressing *Cladosporium fulvum Avr2* (*At-Avr2-A*) and corresponding progenitor Col-0 plants, upon treatment with the biotinylated cysteine protease inhibitor DCG-04 and isolation using streptavidin-coated beads. Active cysteine proteases are detected with streptavidin coupled horseradish peroxidase. Prior to labeling with DCG-04, the extracts received no treatment (-), or were treated with either E-64 or Avr2.

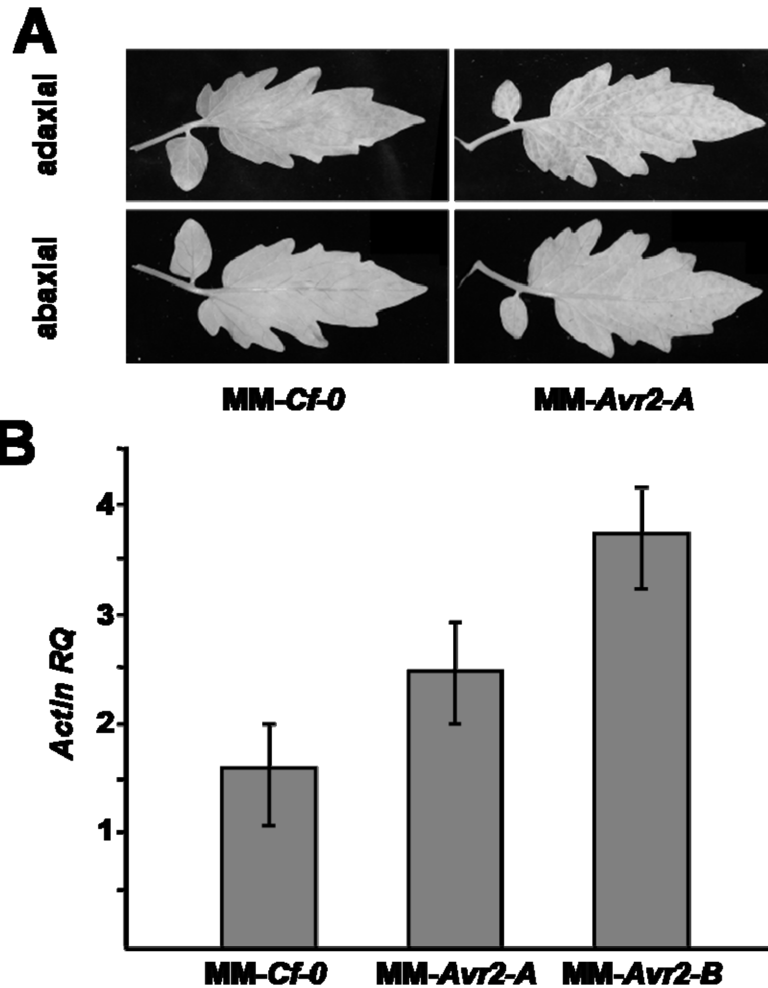


Figure 3. ***Avr2*****-Expressing Tomato Is More Susceptible to Race 2** ***Cladosporium fulvum*****.**
(A) Typical disease symptoms on the adaxial and abaxial sides of the leaflets after inoculation with a race 2 *C. fulvum* strain of *Avr2*-expressing tomato (MM-*Avr2-A*), compared to those of the the progenitor line (MM-*Cf-0*) at 11 DPI. The adaxial side of MM-*Avr2-A* shows enhanced *C. fulvum*-induced chlorosis when compared to MM-*Cf-0*. On the abaxial side, a pale yellowish shade around the main veins indicative of fungal growth is observed on MM-*Avr2-A* and not on MM-*Cf-0*.
(B) Quantitative real-time PCR of fungal colonization levels (*Actin RQ*) determined by comparing *C. fulvum* actin transcript levels (a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) on MM-*Avr2-A* plants when compared to the progenitor line (MM-*Cf-0*) at 10 and 14 DPI. The MM-*Cf-0* at 10 DPI is set to 1. Bars represent the average and standard deviation of minimum three measurements and different letters above the data points represent significant ($P < 0.05$) differences between means.

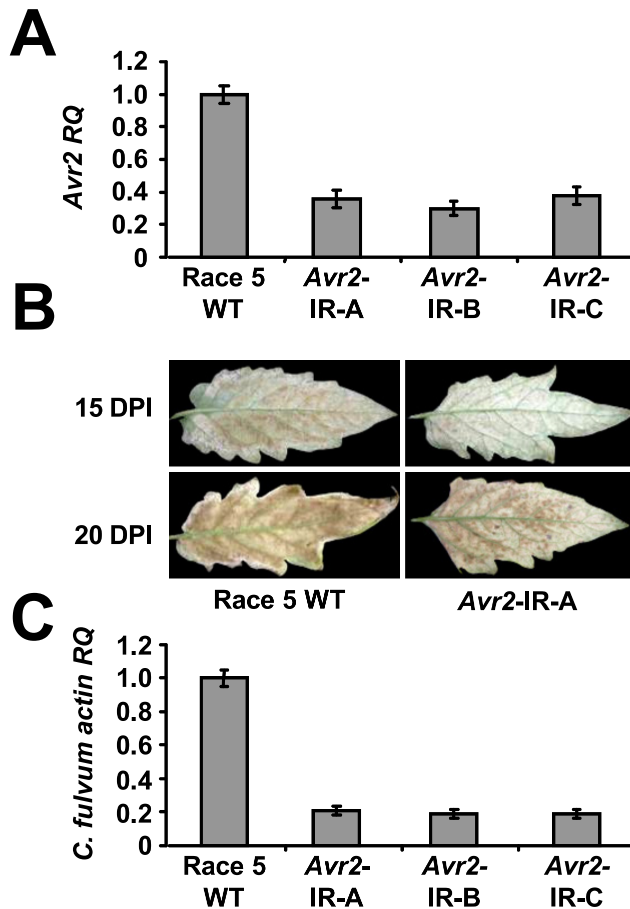


Figure 4. Silencing of *Avr2* in *Cladosporium fulvum* Decreases Virulence on Tomato.

(A) Quantitative real-time PCR of *Avr2* transcript levels (*Avr2 RQ*) of a virulent wild-type (WT) race 5 strain and three independent *Avr2*-silenced race 5 strains on MM-*Cf-0* tomato plants. *Avr2* transcript levels are shown in three independent *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to -C) relative to *C. fulvum* actin transcript levels (for equilibration) when compared to the progenitor strain (Race 5 WT, set to 1) at 11 DPI. Different letters above the data points represent significant ($P < 0.05$) differences between means.

(B) Typical disease symptoms after inoculation of MM-*Cf-0* tomato plants with the *Avr2*-silenced *C. fulvum* transformant *Avr2*-IR-A, as a representative example, when compared to the progenitor strain (Race 5 WT), monitored at 15 and 20 DPI.

(C) Quantitative real-time PCR of fungal colonization (*C. fulvum actin RQ*) by comparing *C. fulvum* actin transcript levels (as a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) for three independent *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to -C) when compared to the progenitor strain (Race 5 WT, set to 1) at 11 DPI. Different letters above the data points represent significant ($P < 0.05$) differences between means.

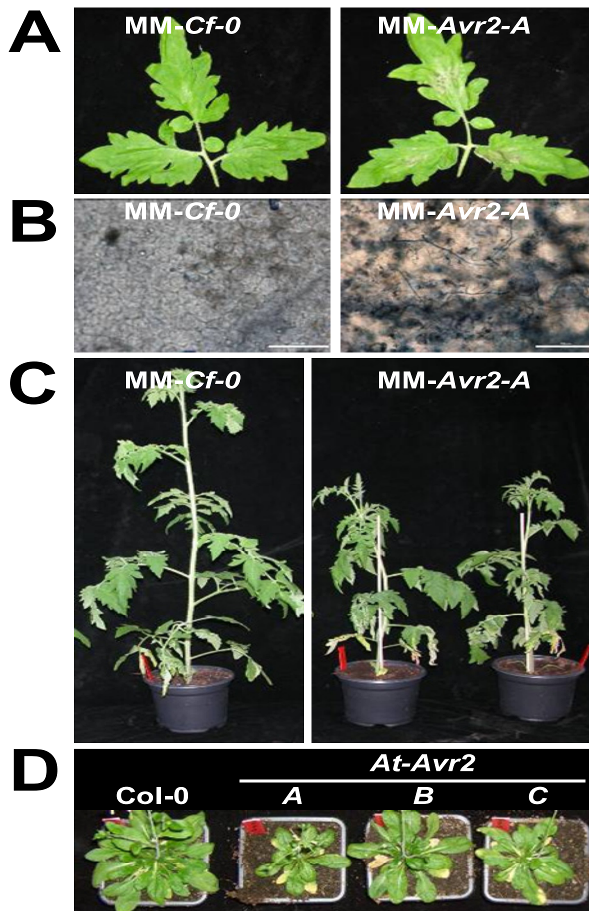


Figure 5. *Avr2*-Expressing Plants Are More Susceptible to *Verticillium dahliae* and *Botrytis cinerea*.

(A) Typical appearance of *Avr2*-expressing tomato leaves (MM-*Avr2-A*) when compared to the progenitor line (MM-*Cf-0*) upon inoculation with *B. cinerea* at three DPI.

(B) Microscopic observation of *Avr2*-expressing cleared tomato leaves (MM-*Avr2-A*) when compared to the progenitor line (MM-*Cf-0*) upon inoculation with *B. cinerea* at two DPI after staining of fungal hyphae and dead plant cells with Trypan blue.

(C) Typical appearance of *Avr2*-expressing tomato plants (MM-*Avr2-A*) when compared to the progenitor line (MM-*Cf-0*) upon inoculation with *V. dahliae* at two weeks post inoculation.

(D) Typical stunting induced by *V. dahliae* on three independent *Avr2*-expressing Arabidopsis lines (*At-Avr2-A* to *-C*) when compared to the progenitor line (Col-0) at two weeks post inoculation.

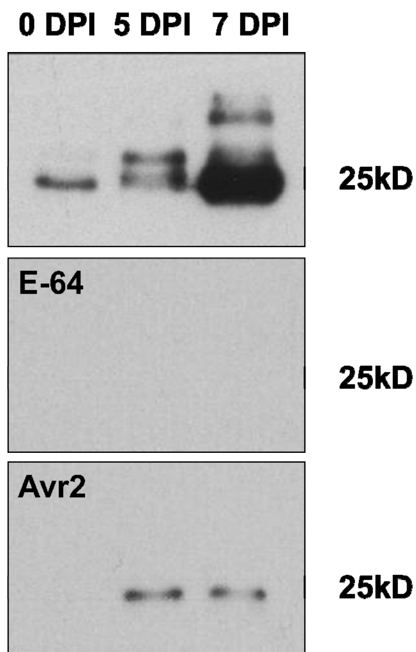
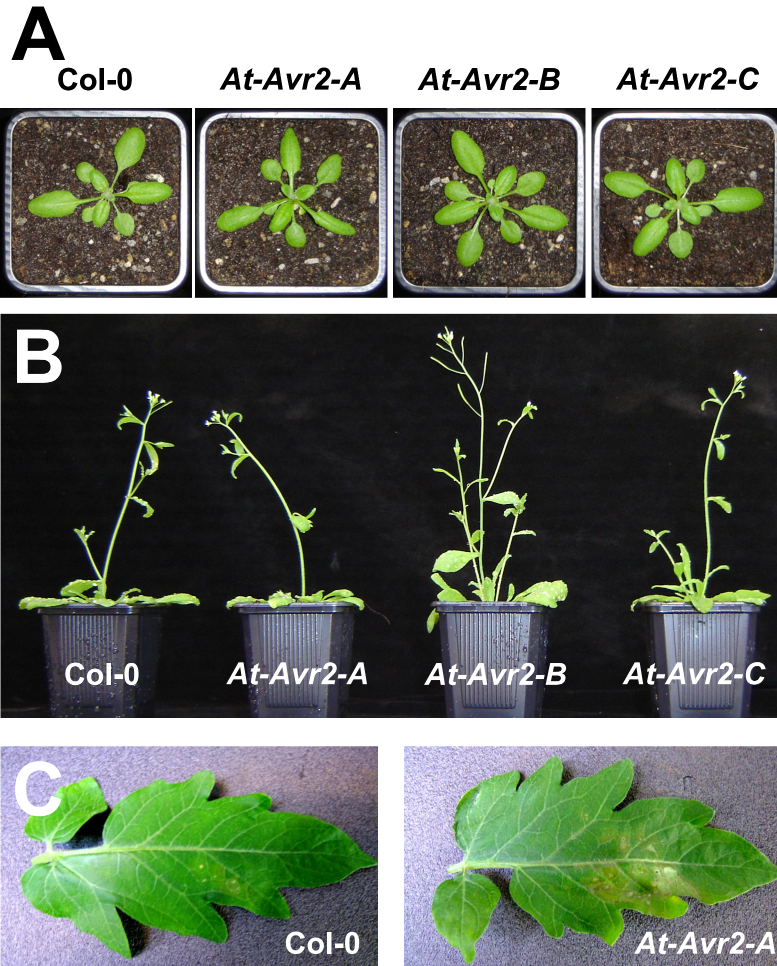
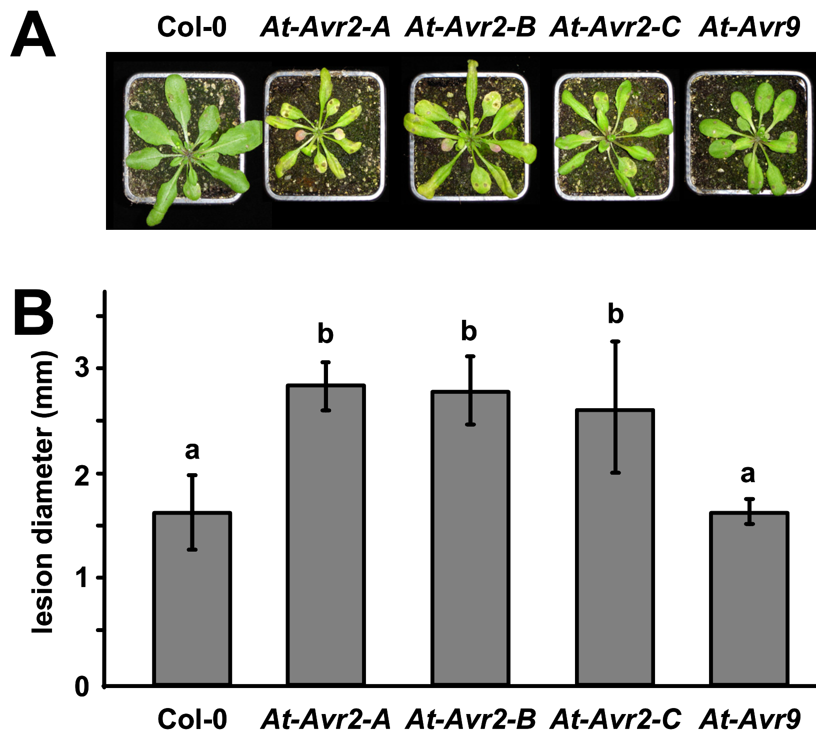


Figure 6. Active Cysteine Proteases Accumulate in the Tomato Apoplast Isolated From *Cladosporium fulvum*-Inoculated Leaves.

Western blot of apoplastic fluids from tomato plants upon inoculation with *C. fulvum* at 0, 5 and 7 DPI upon treatment with the biotinylated cysteine protease inhibitor DCG-04 and isolation using streptavidin-coated beads. Active cysteine proteases were detected with streptavidin-coupled horseradish peroxidase (HRP). Prior to labeling with DCG-04, the extracts received no treatment (upper panel), or were treated with E-64 (middle panel) or Avr2 (lower panel).



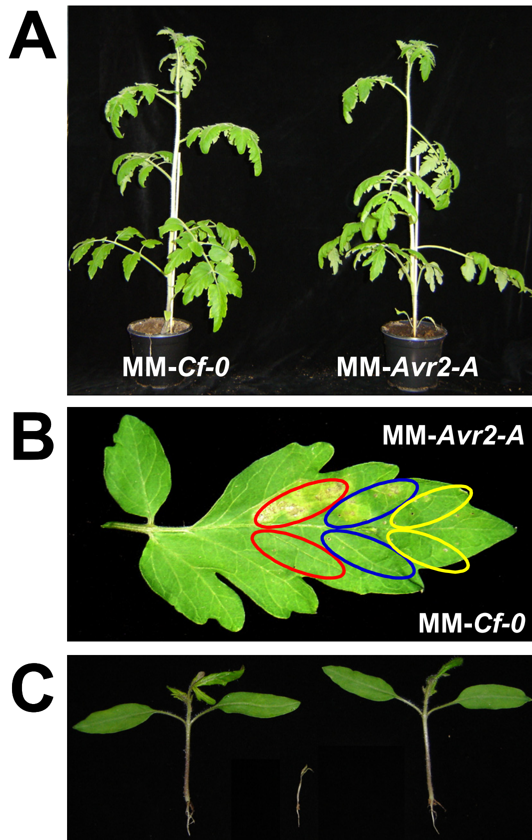
Supplemental Figure 1. Characterization of *Avr2*-Expressing Arabidopsis Plants.
(A, B) Typical appearance of three-week-old (A) and six-week-old (B) plants from three independent *Avr2*-expressing Arabidopsis lines (*At-Avr2-A* to *-C*) when compared to the progenitor line (Col-0).
(C) Production of Avr2 in transgenic Arabidopsis plants (line *At-Avr2-A* as a representative example, right panel) when compared to the progenitor line (Col-0, left panel) demonstrated by injection of apoplastic fluid from those plants (total protein content of 1.3 µg/µL) into leaves of MM-*Cf-2* tomato. The photograph was taken four days post injection.



Supplemental Figure 2. *Avr2*-Expressing Arabidopsis Is More Susceptible to the Fungal Pathogen *Botrytis cinerea*.

(A) Typical symptoms caused by *B. cinerea* on four-week-old plants of three independent *Avr2*-expressing Arabidopsis lines (*At-Avr2-A* to *-C*) at four DPI. Typical symptoms on the progenitor Col-0 line and an *Avr9*-expressing transgenic line (*At-Avr9*) are shown as controls.

(B) Average lesion diameter caused by *B. cinerea* on four-week-old plants of three independent *Avr2*-expressing Arabidopsis lines (*At-Avr2-A* to *-C*), the progenitor Col-0 line, and an *Avr9*-expressing transgenic line (*At-Avr9*) at four DPI. Bars represent the mean with standard deviation and different letters indicate significant differences ($P < 0.05$).

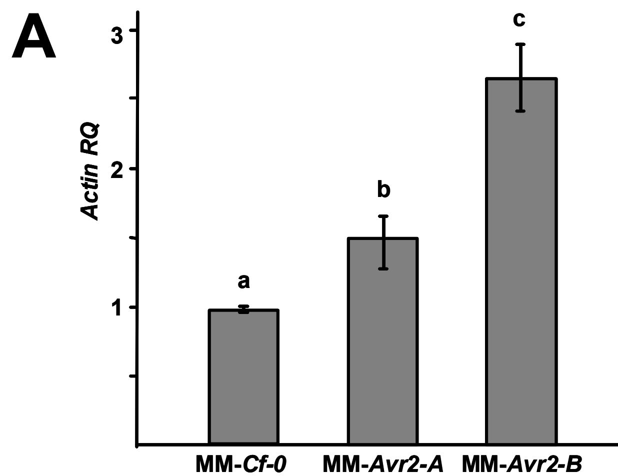


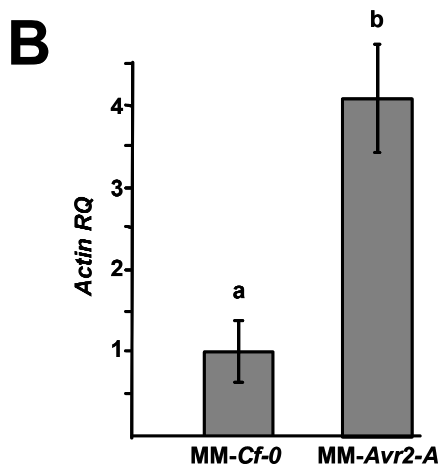
Supplemental Figure 3. Characterization of *Avr2*-Expressing Tomato Plants.

(A) Typical appearance of four-week-old plants from an *Avr2*-expressing tomato line (MM-*Avr2*) when compared to the progenitor line (MM-*Cf-0*).

(B) Production of Avr2 in transgenic tomato (MM-*Avr2-A*), but not in the progenitor line (MM-*Cf-0*) demonstrated by injection of apoplastic fluid into a leaf of MM-*Cf-2* tomato. A dilution series was made from a single stock containing 0.6 (red), 0.2 (blue), or 0.07 (yellow) µg total protein/µl, and the photograph was taken four days post injection.

(C) Production of Avr2 in transgenic tomato (MM-*Avr2-A*) demonstrated by crossing to MM-Cf-2 tomato. The cross resulted in viable seeds, but seedlings die soon after germination, around the time of appearance of the cotelydons. Seedlings from left to right: MM-*Avr2-A*, progeny of cross and MM-*Cf-2*.

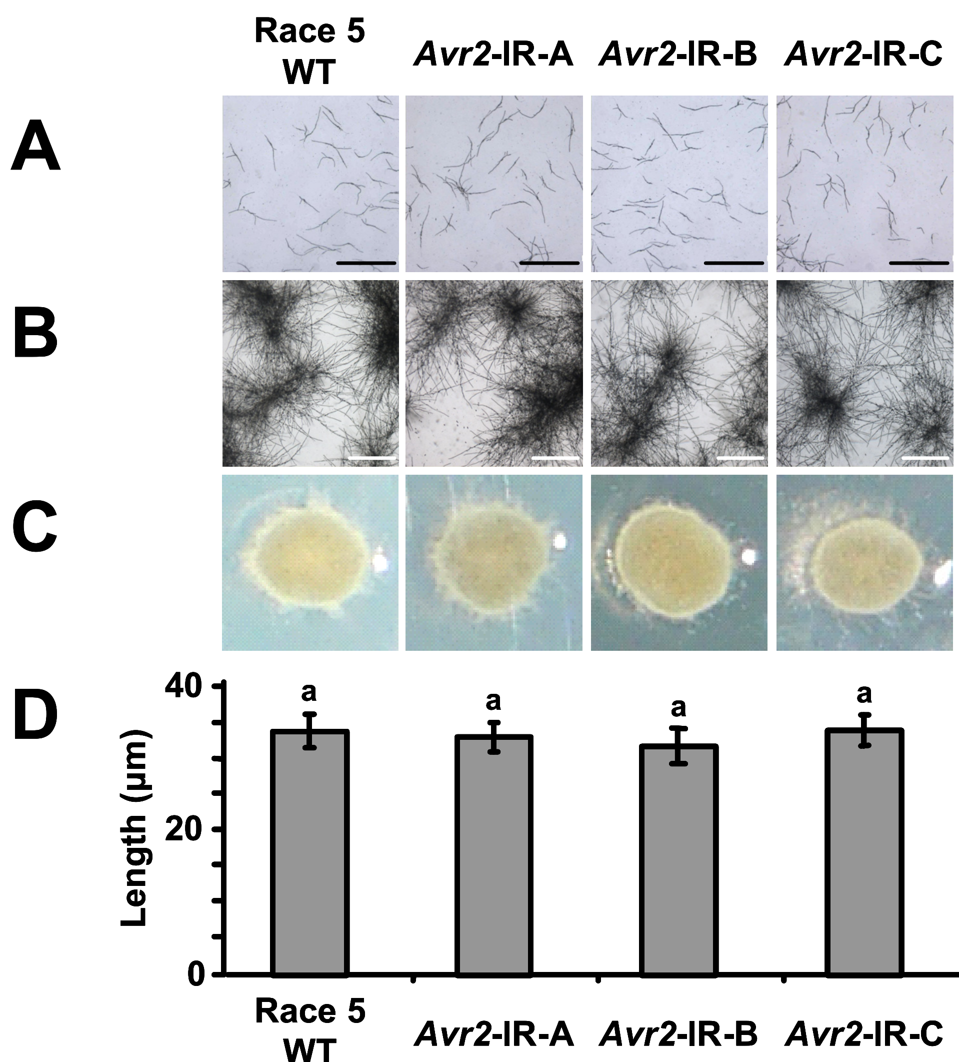




Supplemental Figure 4. *Avr2*-Expressing Tomato Is More Susceptible to Race 2 *Cladosporium fulvum*.

(A) Quantitative real-time PCR of fungal colonization levels (*Actin RQ*) by a race 2 *C. fulvum* strain (race 2.4.5) determined by comparing *C. fulvum* actin transcript levels (a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) on two independent *Avr2*-expressing tomato lines (MM-*Avr2-A* and MM-*Avr2-B*) when compared to the progenitor line (MM-*Cf-0*, set to 1) at 11 DPI. Bars represent the average and standard deviation of minimum three measurements and different letters above the data points represent significant ($P < 0.05$) differences between means.

(B) Quantitative real-time PCR of fungal colonization levels (*Actin RQ*) determined by comparing *C. fulvum* actin transcript levels (a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) MM-*Avr2-A* when compared to the progenitor line (MM-*Cf-0*, set to 1) when inoculated with another race 2 *C. fulvum* strain (race 2.5.9) at 20 DPI. Bars represent the average and standard deviation of minimum three measurements and different letters indicate significant differences ($P < 0.05$).



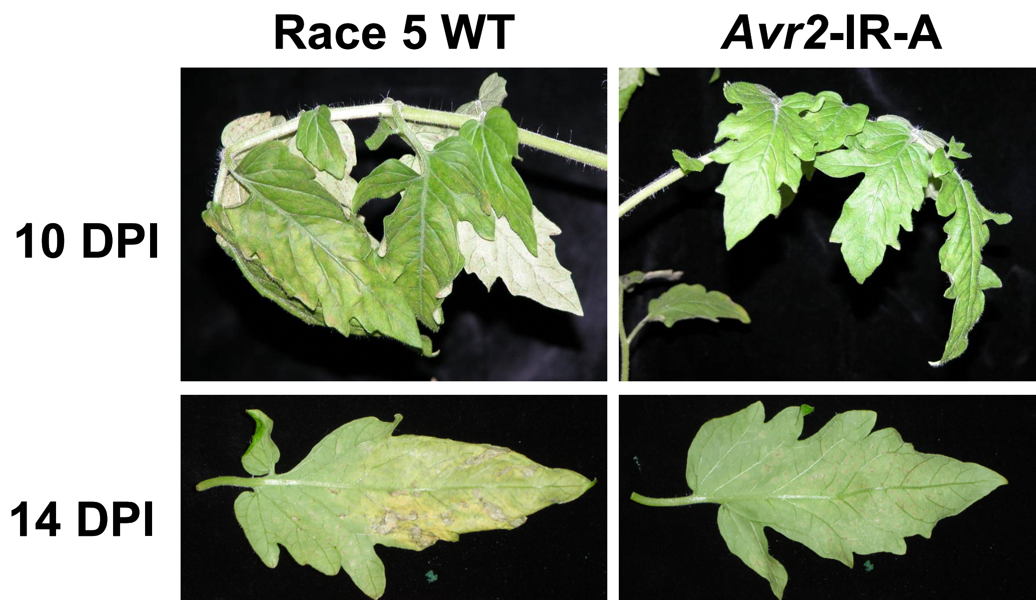
Supplemental Figure 5. Silencing of *Avr2*-Expression in *Cladosporium fulvum* Does not Compromise *in Vitro* Growth.

(A) Micrograph of germinating *C. fulvum* conidia of the *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to –C) when compared to the progenitor strain (Race 5 WT) grown on potato dextrose agar (PDA) for 24 hours. The black bar indicates 50 µM.

(B) Micrograph of germinating *C. fulvum* conidia of the *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to –C) when compared to the progenitor strain (Race 5 WT) grown on PDA for 4 days. The white bar indicates 50 µM.

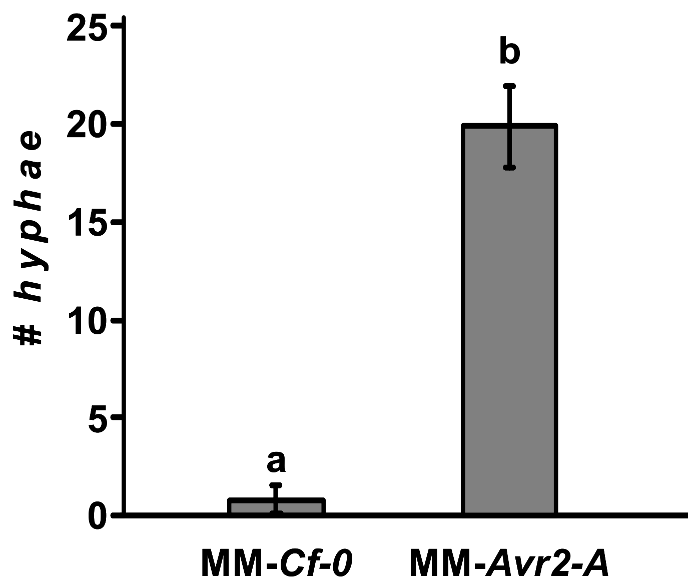
(C) Single spore culture of the *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to –C) when compared to the progenitor strain (Race 5 WT) after 14 days of culturing on PDA.

(D) Average hyphal length of germinating conidia after 24 hours on PDA for the *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to –C) when compared to the progenitor strain (Race 5 WT). Bars represent the average and standard deviation for 30 conidia and different letters indicate significant differences ($P < 0.05$).

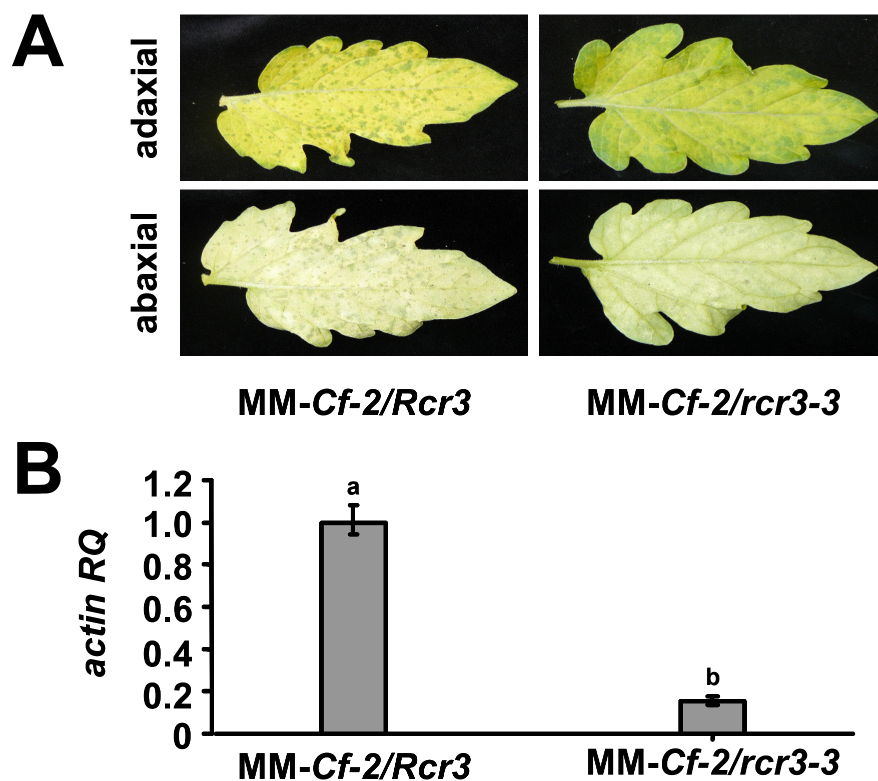


Supplemental Figure 6. Silencing of *Avr2*-Expression in *Cladosporium fulvum* Compromises Cf-2-Mediated Immunity.

Typical appearance of MM-*Cf-2* upon inoculation with the *Avr2*-silenced *C. fulvum* transformant *Avr2*-IR-A, as a representative example, when compared to the progenitor strain (Race 5 WT), monitored at 10 and 14 DPI. Note the strong epinastic response upon inoculation with the progenitor strain, but not upon inoculation with the *Avr2*-silenced transformant (upper panels).



Supplemental Figure 7. *Avr2*-Expressing Plants Are More Susceptible to *Botrytis cinerea*.
Fungal colonization measured by counting of the number of elongated hyphae (> 100 µm) upon microscopic observation of *Avr2*-expressing cleared tomato leaves (MM-*Avr2-A*) when compared to the progenitor line (MM-*Cf-0*) after inoculation with *B. cinerea* at two DPI after staining of fungal hyphae with Trypan blue. Bars represent the average and standard deviation of minimum three measurements and different letters indicate significant differences ($P < 0.05$).



Supplemental Figure 8. *Cf-2* Tomato Lacking Rcr3 Is Not More Susceptible to Race 2 *Cladosporium fulvum*.

(A) Typical disease symptoms on the adaxial and abaxial sides of MM-*Cf-2/Rcr3* tomato and the mutant MM-*Cf-2/rcr3-3* that is devoid of wild-type Rcr3 at 14 DPI. The adaxial side of MM-*Cf-2/Rcr3* shows enhanced *C. fulvum*-induced chlorosis when compared to MM-*Cf-2/rcr3-3*. On the abaxial side, more *C. fulvum* growth is observed on MM-*Cf-2/Rcr3* than on MM-*Cf-2/rcr3-3*.

(B) Quantitative real-time PCR of fungal colonization levels (*Actin RQ*) determined by comparing *C. fulvum* actin transcript levels (a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) on MM-*Cf-2/rcr3-3*plants when compared to the progenitor line (MM-*Cf-2/Rcr3*, set to 1) at 14 DPI. Bars represent the average and standard deviation of minimum three measurements and different letters above the data points represent significant ($P < 0.05$) differences between means.

Supplemental Table 1. Primers Used In This Study.

Primer name	Sequence (5’-3’)[1]	Description
PR1a-ClaI-F	**ATCGAT**ATGGGATTTGTTCTCTTTTCACAATTG	PR1a signal sequence (*Cla*I)
PR1a-Avr2-F	CACTCTTGCCGTGCCCAAAATGCCAAAAAACTACCTGGCTGC	PR1a-Avr2 fusion
Avr2-PR1a-R	GCAGCCAGGTAGTTTTTTGGCATTTTGGGCACGGCAAGAGTG	PR1a-Avr2 fusion
Avr2-R	TCAACCGCAAAGACCAAAACAG	*Avr2* expression
PR1a-Avr4E-F	CACTCTTGCCGTGCCCAAAATGATTTCTCGCGCGATTGCCCGC	PR1a-Avr4E fusion
Avr4E-PR1a-R	GCGGGCAATCGCGCGAGAAATCATTTTGGGCACGGCAAGAGTG	PR1a- Avr4E fusion
Avr4E-R	CTATCTGTTTGCCATCCTCTC	*Avr4E* expression
PR1a-Ecp2-F	CACTCTTGCCGTGCCCAAAATCGGAACGCTGGCAACTCGCCCGGCTC	PR1a-Ecp2 fusion
Ecp2-PR1a-R	GAGCCGGGCGAGTTGCCAGCGTTCCGATTTTGGGCACGGCAAGAGTG	PR1a-Ecp2 fusion
Ecp2-R	CTAGTCATCGTTGGACGGGTTG	*Ecp2* expression
PR1a-Ecp4-F	CACTCTTGCCGTGCCCAAAATGCCGACCCTTCCTTCCGCTTC	PR1a-Ecp4 fusion
Ecp4-PR1a-R	GAAGCGGAAGGAAGGGTCGGCATTTTGGGCACGGCAAGAGTG	PR1a-Ecp4 fusion
Ecp4-R	TTACGGGCAAGTGACCTGCAC	*Ecp4* expression
PR1a-Ecp5-F	CACTCTTGCCGTGCCCAAAATAGGGGCGACAATAAGCCCG	PR1a-Ecp5 fusion
Ecp5-PR1a-R	CGGGCTTATTGTCGCCCCTATTTTGGGCACGGCAAGAGTG	PR1a-Ecp5 fusion
Ecp5-R	CTATCCAGAACTCTGACAC	*Ecp5* expression
oligo-dT	TT**GGATCCTCGAG**TTTTTTTTTTTTTTTTTT	Poly-T (*Nco*I and *Sac*I)
Nco-Avr2-F	TTTTTT**CCATGG**ATGAAGCTCTTCATACTG	*Avr2* inverted repeat (*Nco*I)
Avr2-EcoRI-R2	**GAATTC**ACCGCAAAGACCAAAACAG	*Avr2* inverted repeat (*Eco*RI)
Avr2-NotI-R2	**GCGGCCGC**ACCGCAAAGACCAAAACAG	*Avr2* inverted repeat (*Not*I)
Avr2-(RT)-F	ACCTTCATCTGGCTACTTAC	*C. fulvum Avr2*
Avr2-(RT)-R	CGCAAAGACCAAAACAGC	*C. fulvum Avr2*
CF-GAPDH-F	GGAAACCGGAACCGTTCAG	*C. fulvum* actin
CF-GADPH-R	TGTTAGTGATCCCTTGTGATCCAA	*C. fulvum* actin
CF-Act	CATCGGCAACGAGCGATT	Tomato actin
CF-Act	TGGTACCACCAGACATGACAATG	Tomato actin

Table 1. Gene Set Enrichment Analysis (GSEA) of the Transcriptome of Four-Week-Old Unchallenged *Avr2*-Expressing Arabidopsis Plants.

Description of gene set	Size[a]	Tag[b]	P-value[c]	FDR q-value[d]	Reference
Type III-induced genes of *Pseudomonas syringae* coronatine mutant	264	38%	0.000	0.000	Thilmony et al., 2006; Table S5
P. syringae type III-induced genes	263	38%	0.000	0.000	Thilmony et al., 2006; Table S7
SNARE interactions in vesicular transport	46	63%	0.000	0.000	KEGG pathway
Ndr1-specific upon challenge with *P. syringae* expressing *AvrRpt2*	50	42%	0.000	0.001	Sato et al., 2007
Alternaria (AAL) toxin-induced programmed cell death at 72 hr	92	25%	0.000	0.001	Gechev et al., 2004
N-glycan biosynthesis	20	60%	0.003	0.003	KEGG pathway
Alternaria (AAL) toxin-induced programmed cell death at 48 hr	92	24%	0.000	0.005	Gechev et al., 2004
Alternaria (AAL) toxin-induced programmed cell death at 24 hr	87	21%	0.003	0.024	Gechev et al., 2004
Nucleotide sugars metabolism	15	20%	0.029	0.047	KEGG pathway
P. syringae coronatine-regulated genes	323	28%	0.000	0.053	Thilmony et al., 2006; Table S6
Salicylic acid-repressed auxin signalling pathway	20	45%	0.036	0.075	Wang et al., 2007
Glycan biosynthesis	19	47%	0.056	0.142	KEGG pathway
Ribosome	180	59%	0.008	0.134	KEGG pathway
PAMP-repressed genes	115	28%	0.019	0.137	Thilmony et al., 2006; Table S2
Proteasome	40	40%	0.101	0.223	KEGG pathway
E. coli strain O157:H7-induced genes	220	33%	0.042	0.253	Thilmony et al., 2006
Metabolism of xenobiotics by cytochrome p450	17	47%	0.157	0.270	KEGG pathway
Alternaria (AAL) toxin-induced programmed cell death at 7 hr	55	18%	0.110	0.260	Gechev et al., 2004
Fusarium oxysporum Nep1 toxin-induced death	432	15%	0.047	0.323	Bae et al., 2006
P. syringae regulated genes	154	27%	0.129	0.369	Thilmony et al., 2006; Table S2

[a]Number of genes present in the gene set or KEGG pathway after filtering out those genes not in the expression dataset.

[b]The percentage of gene hits before or after the peak in the running enrichment score, gives an indication of the percentage of genes contributing to the enrichment score.
[c]Nominal p-value, the statistical significance of the enrichment score.
[d]False discovery rate q-value.

Table 2. Active Cysteine Proteases Identified in Total Extracts of Arabidopsis Plants.

Probe		**DCG-04 (biotinylated E-64)**					
Treatment		**Non-inoculated**					
Plant Genotype		**Col-0**			***At-Avr2-A***		
Competitor		**No competitor**	**Excess E-64**	**Excess Avr2**	**No competitor**	**Excess E-64**	**Excess Avr2**
Protease	XCP1 (O65493) [1]	**4** (2-0-1-1-0)[4]	-	-	-	-	
	XCP2 (Q9LM66)	**11** (6-3-1-1-0)	-	-	-	-	
	Cathepsin B (Q9ZSI0)[2]	**12** (10-1-1-0-0)	-	**10** (6-1-1-1-1)	**10** (10-0-0-0-0)	-	**11** (11-0-0-0-0)
	RD21A (P43297)	**13** (12-1-0-0-0)	-	-	**10** (9-1-0-0-0)	-	**3** (3-0-0-0-0)
	CPR1 (Q9LT77)[3]	**5** (2-1-1-1-0)	-	-	-	-	
	Aleurain (Q8H166)	**9** (7-2-0-0-0)	-	-	**21** (18-1-0-1-1)	-	**21** (19-0-0-1-1)
	Aleurain-like (Q8RWQ9)	**7** (5-1-0-0-1)	-	-	**4** (4-0-0-0-0)	-	**8** (7-1-0-0-0)

[1] Codes in between brackets are TrEMBL entry codes.
[2] The same peptides were identified for Cathepsin B (Q94K85).
[3] The same peptides were identified for pseudotzain (Q3EB42).
[4] Numbers in bold correspond to the amount of peptides observed that belong to the corresponding cysteine protease. Numbers in brackets show the position of the peptide in the cross-correlation scan list (from first through fifth rank, respectively). The filtering criteria were as described by Peng et al. (2003).

Table 3. Active Cysteine Proteases Identified in Total Extracts of Tomato Plants.

Probe		**DCG-04 (biotinylated E-64)**							**biotinylated Avr2**	
Treatment		**Non-inoculated**					***C. fulvum* race 2**		**Non-inoculated**	
Plant genotype		**MM-*Cf-0***			**MM-*Avr2-A***		**MM-*Cf-0***		**MM-*Cf-0***	
Competitor		**No competitor**	**Excess E-64**	**Excess Avr2**	**No competitor**	**Excess E-64**	**No competitor**	**Excess E-64**	**No competitor**	**Excess E-64**
Protease	Rcr3 (TC128871)[1]	**5** (4-1-0-0-0)[2]	**-**	**-**	**-**	**-**	**13** (8-1-1-2-1)	**-**	**7** (6-1-0-0-0)	**-**
	Pip1 (TC118154)	**17** (17-0-0-0-0)	**-**	**-**	**7** (6-1-0-0-0)	**-**	**53** (49-2-1-1-0)	**-**	**7** (7-0-0-0-0)	**-**
	Cathepsin B (TC162008)	**4** (4-0-0-0-0)	**-**	**3** (3-0-0-0-0)	**6** (6-0-0-0-0)	**-**	**3** (3-0-0-0-0)	**-**	**-**	**-**
	Cathepsin B (TC162009)	**9** (9-0-0-0-0)	**-**	**7** (7-0-0-0-0)	**11** (11-0-0-0-0)	**-**	**4** (4-0-0-0-0)	**-**	**-**	**-**
	TDI65 (TC124125)	**15** (15-0-0-0-0)	**-**	**7** (6-0-1-0-0)	**18** (17-1-0-0-0)	**-**	**18** (12-3-3-0-0)	**-**	**3** (3-0-0-0-0)	**-**
	Aleurain (TC116458)	**11** (11-0-0-0-0)	**-**	**9** (9-0-0-0-0)	**12** (12-0-0-0-0)	**-**	**13** (10-2-0-1-0)	**-**	**2** (2-0-0-0-0)	**-**
	Glycinain (TC124017)	**3** (2-1-0-0-0)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

[1] Codes in between brackets are TIGR's Tentative Consensus sequences.

[2] Numbers in bold correspond to the amount of peptides observed that belong to the corresponding cysteine protease. Numbers in brackets show the position of the peptide in the cross-correlation scan list (from first through fifth rank, respectively). The filtering criteria were as described by Peng et al. (2003).

CHAPTER 4

Affinity of Avr2 for tomato cysteine protease Rcr3 is positively correlated with the Avr2-triggered Cf-2-mediated hypersensitive response

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Submitted

Abstract
The *Cladosporium fulvum* Avr2 effector is a novel type of cysteine protease inhibitor with eight cysteine residues that are all involved in disulfide bonds. We have produced wild type Avr2 protein in *Pichia pastoris* and determined its disulfide bond pattern. By site-directed mutagenesis of all eight cysteine residues we show that three of the four disulfide bonds are required for Avr2 stability. The six C-terminal amino acid residues contain a disulfide bond that is not embedded in the overall Avr2 structure. Avr2 is not processed by the tomato cysteine protease Rcr3 and behaves as an uncompetitive inhibitor. Subsequently, we produced mutant Avr2 proteins in which selected amino acid residues were individually replaced by an alanine, or all six C-terminal amino acid residues were deleted. Of these Avr2 mutants we determined their affinity (K_i) for Rcr3 and their ability to trigger a Cf-2-mediated hypersensitive response (HR) in tomato. We found that the two C-terminal cysteine residues and the six amino acid C-terminal tail of Avr2 are required for Rcr3-inhibitory activity and the ability to trigger a Cf-2-mediated HR. Individual replacement of Lys17, Lys20, Tyr21 and Phe54 by alanine did not significantly affect the biological activity of Avr2. Overall, our data suggest that the affinity of the Avr2 mutants for Rcr3 is positively correlated with their ability to trigger a Cf-2-mediated HR.

Introduction

Cladosporium fulvum (syn. *Passalora fulva*) is an asexual fungal pathogen causing leaf mould of tomato that complies with the gene-for-gene relationship (De Wit et al., 2009; Stergiopoulos and De Wit, 2009; Thomma et al., 2005). The fungus penetrates tomato leaves through stomata and colonizes the apoplastic space. During infection and subsequent colonization of tomato the fungus secretes several small cysteine-rich effectors known as Avrs (Avirulence proteins) and Ecps (extracellular proteins). The genes encoding these effectors have been cloned and for a number of them a role in fungal virulence has been demonstrated (Bolton et al., 2008; De Wit et al., 2008; Stergiopoulos and De Wit, 2009; Thomma et al., 2005; Van Esse et al., 2007) Resistant tomato plants contain *Cf* genes that recognize the presence or activity of their cognate effectors and mediate an array of defense responses culminating in the hypersensitive response (HR) (De Wit et al., 2008). The *Cf-2, Cf-4*, *Cf-4E* and *Cf-9* genes which mediate recognition of Avr2, Avr4, Avr4E and Avr9, respectively, have been cloned (Dixon et al., 1996; Jones et al., 1994; Takken et al., 1999; Thomas et al., 1997).

The Avr2 effector is a virulence factor of *C. fulvum* that inhibits tomato cysteine proteases including Rcr3, Pip1, aleurain and TDI-65 which are important for basal host defense (Krüger et al., 2002; Rooney et al., 2005; Schipper et al., 2009; Shabab et al., 2008; Van Esse et al., 2008). Avr2 can also increase the virulence of other fungal pathogens such as *Botrytis cinerea* and *Verticillium dahliae*, as could be shown on tomato and *Arabidopsis thaliana* transgenic for *Avr2* (Van Esse et al., 2008). In the presence of Cf-2, Avr2 behaves as an avirulence factor that triggers Cf-2-mediated HR that also requires Rcr3^{pim} (required for C, *fulvum* resistance), a cysteine protease originating from *Lycopersicon pimpinellifolium* (Krüger et al., 2002; Rooney et al., 2005).

The Avr4 effector is a virulence factor of *C. fulvum* with a functional chitin-binding domain that protects chitin present in fungal cell walls against plant chitinases (Van den Burg et al., 2006; Van den Burg et al., 2004; Van den Burg et al., 2003; Van Esse et al., 2007). In the presence of Cf-4, Avr4 behaves as an avirulence factor that triggers Cf-4-mediated HR (Joosten et al., 1994).

The virulence function of Avr4E is not known yet. In the presence of Cf-4E, Avr4E behaves as an avirulence factor that triggers Cf-4E-mediated HR (Westerink et al., 2004).

The Avr9 effector contains a cystine knot with structural but, so far, no functional homology to carboxypeptidase inhibitor (Van den Ackerveken et al., 1993; Van den Hooven et al., 2001; Van Kan et al., 1991; Vervoort et al., 1997). Disruption of *Avr9* in *C. fulvum* did not affect virulence on tomato plants, suggesting that it is not required for full virulence (Marmeisse et al., 1993). Expression of *Avr9 in vitro* is induced under nitrogen-limiting conditions (Pérez-Garcia et al., 2001; Thomma et al., 2006; Van den Ackerveken et al., 1994) and an *Nrf1* gene (for nitrogen responsive factor)

has been identified in *C. fulvum*. *Nrf1* deletion mutants no longer express *Avr9* under nitrogen-limiting conditions *in vitro* and are compromised in their virulence on Cf-0 tomato plants (Thomma et al., 2006). However, these mutants are still avirulent on Cf-9 tomato plants, suggesting that Nrf1 is a major, but not the only positive regulator of *Avr9* expression (Pérez-Garcia et al., 2001). Expression of all other known *Avr* and *Ecp* effector genes of *C. fulvum* is not induced under nitrogen-limiting conditions (Bolton et al., 2008; Thomma et al., 2006) indicating that nitrogen-limitation is not a general environmental condition that induces effector genes of *C. fulvum* (Bolton and Thomma, 2008).

All Avr effectors trigger Cf-mediated HR, which could suggest a direct interaction between effector and Cf receptor protein. However, using different biochemical approaches direct interaction between the Cf-9 protein and the Avr9 effector could not been shown (Luderer et al., 2001). Avr9 binds to a high-affinity binding site (HABS) that could represent a virulence target that is guarded by the Cf-9 protein (Kooman-Gersmann et al., 1996; Kooman-Gersmann et al., 1998). Expression of the *Avr4* gene in tomato plants without the *Cf-4* gene does not induce or suppress expression of host genes suggesting that Avr4 is a defensive virulence factor that protects the penetrating fungus against deleterious effects of plant chitinases rather than attacking the host plant itself (Van Esse et al., 2009). As plants containing the Cf-4 resistance protein respond with a strong HR, a direct interaction between Avr4 and Cf-4 mediating this response is suggested, although not yet confirmed experimentally.

The interaction between Avr2 and Rcr3 triggers Cf-2-mediated HR which fits the guard hypothesis with Rcr3 as the guardee and Cf-2 as the guard that becomes alerted by Rcr3 after binding to Avr2 (Van der Biezen and Jones, 1998). We have previously shown that Avr2 binds to, and inhibits, the cysteine protease Rcr3, although the detailed mechanism of action is not fully understood (Rooney et al., 2005).

Cysteine proteases are endopeptidases that use a cysteinyl thiol group for their catalytic activity and belong to the C-clan that harbors several families (Rawlings et al., 2004). The majority of the members of the CA-1 subfamily of cysteine proteases is of plant origin and is referred to as papain-like cysteine proteases (PLCPs) as they show similarity to papain, a well studied cysteine protease present in latex of papaya (*Carica papaya*) (Van der Hoorn, 2008). PLCPs are involved in important physiological and cellular processes, including organogenesis, the turnover of storage proteins, programmed cell death, tolerance to abiotic stress, and defense against herbivorous predators and microbial pathogens (Hatsugai et al., 2004; Kiyosaki et al., 2009; Van der Hoorn, 2008). PLCPs have similar folds containing a cysteine and histidine residue in their catalytic centre, which consists of seven subsites (S_1 to S_4 and S'_1 to S'_3) each accommodating a side-chain of a corresponding amino acid residue (P_1 to P_4 and P'_1 to P'_3) of the peptide substrate or

peptide inhibitor (Turk et al., 1998) (Figure 1). The peptide bond of the substrate is cleaved between amino acid residue P_1 and P'_1. The S_2 subsite of PLCPs determines the substrate specificity and has a preference for a non-polar bulky hydrophobic amino acid residue in the P_2 subsite of the substrate or peptide inhibitor with a strong preference for phenylalanine or tyrosine and a slightly lower preference for amino acids like valine, leucine or isoleucine, whereas the S_3 and S_1 subsites are conserved (Lecaille et al., 2002; Pauly et al., 2003; Somoza et al., 2000).
Phytocystatins are plant proteinaceous inhibitors of CA-1 subfamily of cysteine proteases (MEROPS peptidase database, http:merops.sanger.ac.uk; (Rawlings et al., 2010). They share three motifs involved in the interaction with their target enzymes including (i) the reactive site QxVxG, (ii) one or two glycine residues in the N-terminal part, and (iii) a tryptophan residue located downstream of the reactive site. Most phytocystatins have a molecular mass between 12 and 16 kDa and lack disulfide bonds (Martinez and Diaz, 2008). Phytocystatins form tight, reversible complexes with cysteine proteases and act as pseudosubstrates that enter the active site cleft of target enzymes and cause inhibition.
The oomycete pathogen *Phytophthora infestans* secretes a number of protease inhibitors with similarity to cystatins including Epic1 and Epic2B that also interact with, and inhibit, Pip1 and Rcr3 (Shabab et al., 2008; Song et al., 2009; Tian et al., 2007).
Based on its amino acid sequence, the *C. fulvum* cysteine protease inhibitor Avr2 cannot be classified as a typical phytocystatin. Phytocystatins lack disulfide bonds, whereas Avr2 contains eight cysteine residues that are likely involved in disulfide bonds as is the case for many other non-cystatin protease inhibitors which may contain between one and five disulfide bonds (Rawlings et al., 2004). It is difficult to classify protease inhibitors based on sequence information as reactive-site residues of inhibitor domains are often not conserved in a way similar to active-site residues present in target proteases. Moreover, some protease inhibitors contain multiple inhibitor domains that can be targeted to different classes of proteases (Rawlings et al., 2004).
However, if we assume that Avr2 enters the active site cleft of Rcr3, with a preference for a non-polar bulky hydrophobic amino acid residue in the P_2 subsite of Avr2 to interact with the S_2 subsite of Rcr3, apart from individually substituting the eight cysteine residues in Avr2 by alanine, individual substitution of Lys17, Lys20, Tyr21 and Phe54 residues by alanine might also affect the inhibitory activity of Avr2.
Here, we report on the overall structure of Avr2 and show that it is a novel type of cysteine protease inhibitor with eight cysteine residues that are all involved in disulfide bonds. We have produced wild type Avr2 protein in *Pichia pastoris* and determined its disulfide bond pattern. By site-directed mutagenesis of the eight cysteine residues we show that three of the four disulfide bonds are required for Avr2 stability. Avr2 is not processed by Rcr3 and it behaves as an uncompetitive inhibitor

of Rcr3 cysteine protease. Subsequently, we produced mutant Avr2 proteins in which four selective amino acid residues were replaced by alanine, or the six C-terminal amino acid residues were deleted. Of these Avr2 mutants we determined their affinity (K_i) for Rcr3 and their ability to trigger a Cf-2-mediated HR. We found that the two C-terminal cysteine residues and the six amino acid C-terminal tail of Avr2 are required for Rcr3 inhibitory activity and the ability to trigger a Cf-2-mediated HR. Individual replacement of Lys17, Lys20, Tyr21 and Phe54 by alanine did not significantly affect the biological activity of Avr2. Overall, our data suggest that the affinity of Avr2 mutants for Rcr3 is positively correlated with their ability to trigger a Cf-2-mediated HR.

Results

Avr2 contains four disulfide bonds.

Wild type Avr2 tagged at the N-terminus with six histidine residues and Flag (His::Flag::Avr2) containing an enterokinase cleavage site behind the Flag signature was produced in a fermentor by *Pichia pastoris* in a similar fashion as described previously (Rooney et al., 2005; Van den Burg et al., 2001) and purified on a Ni-NTA column (Figure 2A). The His-Flag-tag was removed with enterokinase and the reaction products were separated by analytical RP-HPLC in order to obtain mature untagged Avr2. The molecular mass of purified wild type Avr2, as determined by MALDI-TOF MS analysis, was 6085 Daltons, which suggests that all cysteine residues present in wild type Avr2 are involved in intramolecular disulfide bonds. This could be verified by showing that the molecular mass of completely reduced Avr2 increased by eight Dalton (6093 Dalton).

Disulfide mass mapping of Avr2.

In order to determine the disulfide bond pattern of Avr2, disulfide mass mapping using cyanylation and CN-induced cleavage by hydroxide or ammonia was performed as described (Wu and Watson, 1998). First, Avr2 was partially reduced with a 18-fold molar excess of Tris-(2-carboethyl)phosphine hydrochloride (TCEP). Subsequently, Avr2 was cyanylated with a 300-fold molar excess of 1-cyano-4-dimethylamino-pyridium tetrafluoroborate (CDAP), cleaved by ammonia, and the products were separated by RP-HPLC. Two singly reduced cyanylated Avr2 species were obtained in which the presence of disulfide bonds between Cys7-Cys33 and Cys53-Cys57 could be assigned by mass determination after CN-induced cleavage. The disulfide bonds of doubly reduced species could not be assigned in this way, but were determined by a combination of partial reduction with TCEP and alkylation with N-ethylmaleimide (NEM) followed by separation on RP-HPLC and digestion with *Staphylococcus aureus* endoprotease GluC which cleaves directly C-terminal of a glutamic acid residue. Mass determination of the obtained products showed that the two remaining disulfide bonds are present between Cys12-Cys52 and Cys29-Cys43. The complete disulfide bond pattern of Avr2 is shown in Figure 2 B. The disulfide bonds between Cys7-Cys33, Cys12-Cys43 and Cys29-Cys52 in Avr2 provide a very compact and stable structure which is presumably difficult to be digested by extracellular plant proteases that are present in the apoplastic space of tomato leaves. The C-terminal Cys53-Cys57 disulfide bond is not embedded in the overall Avr2 structure and presumably does not contribute significantly to Avr2 stability, but might still be important for Rcr3-inhibitory activity. The overall disulfide bond pattern of Avr2 is different from disulfide patterns reported for two other *C. fulvum* effectors. Avr9 contains a cystin knot structure (Van den Hooven et al., 2001), whereas Avr4 contains a chitin-binding domain structure (Van den Burg et al., 2003). The overall disulfide bond pattern observed in Avr2 also occurs in members of a serine protease-

inhibiting peptide family isolated from arthropods, although the cysteine spacing pattern present in members of this family is different from Avr2 (Simonet et al., 2002).

Production of mutant Avr2 proteins.

We decided to generate mutations in Avr2 by amino acid substitutions and deletions in areas of the protein that might be involved in cysteine protease binding and cysteine protease inhibition. We substituted all cysteine residues individually by alanines and deleted the C-terminal six amino acids. In addition, Tyr20 and Phe54 were selected to be replaced by alanines as they might function as P_2 subsite to occupy the S_2 subsites of Rcr3. Lys17 and Lys20 were chosen as many cystatins are lysine-rich (Martinez and Diaz, 2008). Figure 2C shows an overview of the amino acid substitutions and the C-terminal deletion that were introduced in the various mutant Avr2 proteins through PCR-based site-directed mutagenesis of the *Avr2* gene. Wild type and mutant versions of the *Avr2* gene were cloned into the expression vector pPIC9 and transformed into the yeast *Pichia pastoris*. Wild type and mutant Avr2 proteins produced in *P. pastoris* were purified, analyzed and quantified by SDS-PAGE (Figure 3). Mutant Avr2 proteins with the following amino acid substitutions could be produced in sufficient amounts: Avr2-Lys17>Ala, Avr2-Lys20>Ala, Avr2-Tyr21>Ala, Avr2-Cys53>Ala, Avr2-Phe54>Ala, Avr2-Cys57>Ala and the Avr2 mutant lacking all six C-terminal amino acid residues (Avr2-Cys53Xxx). Substitution of all cysteine residues by alanines, except for residues Cys53 and Cys57, resulted in unstable Avr2 proteins that could not be produced in sufficient amounts in *P. pastoris*. Equal amounts of mutant Avr2 proteins were used for determining their inhibitory activity towards Rcr3 and their ability to trigger a Cf-2-mediated HR.

Inhibitory activity toward Rcr3 of wild type Avr2 and mutant Avr2 proteins.

Previously, it was shown that wild type Avr2 is not a substrate for Rcr3 as the overall mass of wild type Avr2 did not change after prolonged incubation with Rcr3 (Rooney et al., 2005). Thus it is anticipated that Avr2 interacts with Rcr3 by an irreversible trapping or a reversible tight-binding reaction. To determine which type of interaction occurs we performed a Lineweaver-Burk plot analysis. In this analysis we used a constant Rcr3 concentration and varying concentrations of the green fluorescent casein substrate (Bodipy FL-casein) at four different concentrations of Avr2. Avr2 shows an inhibition pattern that is most similar to that of un-competitive inhibition, as both the V_{max} and K_m of Rcr3 are affected at different concentrations of Avr2 (Figure 4). The pattern observed for 40 nM Avr2 is fairly similar to that observed for 20 nM Avr2 indicating that at 20 nM Avr2 nearly all binding are occupied and increasing the avr2 concentration has only minor affects. Uncompetitive inhibition means that Avr2 only binds to the Rcr3-substrate complex outside the active centre. This is also in agreement with the finding that Avr2 is not a substrate of Rcr3.

We also measured the affinity (K_i value) of wild type and the different Avr2 mutants for Rcr3 by measuring the rest proteases activity at constant Bodipy FL-casein substrate concentration after incubation with varying concentrations of wild type or mutant Avr2 peptides for 24 hours. The K_i value for wild type Avr2 was 16.1 nM and the K_i values for the various mutant Avr2 peptides varied between 20 and 1444 nM (Figure 5). Substitution of Cys53, Phe54 or Cys57 by alanines increased the K_i value approximately five-fold, whereas deletion of all six C-terminal amino acid residues increased the K_i value more than 70-fold. Individual substitution of the Lys17, Lys20 or Tyr21 residue in Avr2 by alanines did not significantly change the K_i value towards Rcr3 as compared to wild type Avr2, suggesting that these residues are not crucial for affinity of Avr2 for Rcr3.

Cf-2-mediated HR triggered by wild type and mutants of Avr2

The mutant Avr2 peptides with various affinities for Rcr3 were also assayed for their ability to trigger a Cf-2-mediated HR in tomato. Equal amounts (100 µl of 2µM) were injected in Cf-2 tomato plants and occurrence of the Cf-2-mediated HR was scored 3 days post injection (Figure 6). The Cf-2-mediated HR triggered by wild type Avr2 and various Avr2 mutants varied significantly. Of the cysteine residues in Avr2 that were substituted by alanines, only results for Cys53>Ala and Cys57>Ala substitutions could be obtained as from the Avr2 mutants in which the individual substitutions Cys7>Ala, Cys12>Ala, Cys29>Ala, Cys33>Ala, Cys43>Ala and Cys52>Ala were introduced no sufficient amounts of Avr2 mutant proteins could be produced in *P. pastoris*, most likely due to reduced protein stability. The Cf-2-mediated HR-inducing activities of Avr2 mutant peptides in which one amino acid residue in the C-terminal part was substituted by an alanine (Avr2-Cys53>Ala, Avr2-Cys57>Ala and Avr2-Phe54>Ala mutants), were significantly lower when compared with the activity of the Avr2 wild type. Deletion of all six C-terminal amino acid residues in Avr2 abolished Cf-2-mediated HR-inducing activity nearly completely.

Substitution of the amino acid residues Lys17, Lys20 or Tyr21 by alanine (Avr2-Lys17>Ala, Avr2-Lys20>Ala and Avr2-Tyr21>Ala) did not significantly change the Cf-2-mediated HR-inducing activity. Altogether these results (shown in Figure 6), combined with the observed K_i values (shown in Figure 5), indicate that there is a positive correlation between the affinity of the Avr2 mutants for Rcr3 and their ability to trigger a Cf-2-mediated HR.

Discussion

MALDI-TOF MS analysis showed that all eight cysteine residues present in the cysteine protease inhibitor Avr2 are involved in disulfide bonds. Disulfide bonds are formed between Cys7-Cys33, Cys12-Cys43, Cys29-Cys52 and Cys53-Cys57. Three of these bonds (Cys7-Cys33, Cys12-Cys43 and Cys29-Cys52) are required for Avr2 stability, and indirectly also for its inhibitory activity against the cysteine protease Rcr3 and its ability to trigger a Cf-2-mediated HR. The spacing pattern between the cysteine residues of Avr2 has not been found so far in protein structure data bases. However, a different cysteine spacing, but similar overall disulfide bond pattern, has been observed in members of a serine protease-inhibiting peptide family isolated from arthropods (Simonet et al., 2002).

Individual substitution of cysteine residues Cys7, Cys12, Cys29, Cys33, Cys43 or Cys52 by alanines strongly affected the stability of the Avr2 protein and this was the reason why we could not produce these Avr2 mutant proteins in sufficient amounts in *P. pastoris* for detailed biological studies. Substitution of the cysteine residues forming the disulfide bond between Cys53 and Cys57 in the C-terminus of Avr2 by alanines did not affect the stability of the corresponding mutant proteins, but showed a reduction in both their Rcr3-inhibitory activity and their ability to trigger a Cf-2-mediated HR. In addition, deletion of the six C-terminal amino acid residues of Avr2 showed a decrease in Rcr3 inhibition and Cf-2-dependent HR-inducing activity of over 100-fold. This indicates that the C-terminal part of Avr2 is crucial for biological activity and most likely interacts with Rcr3. In order to find direct evidence for this interaction we performed MALDI-TOF MS analysis of Rcr3 in the presence of biotinylated Avr2 and tried to detect the Rcr3-Avr2 complex in co-immunoprecipitions in order to determine the site of interaction. However, we were able identify fragments of Rcr3 after trypsin digestion but we could not identify Rcr3-Avr2 fragments indicating that the interaction between Rcr3 and Avr2 is reversible which makes it difficult or even impossible to identify the interaction site between Rcr3 and Avr2 by mass spectrometry.

Amino acid substitutions outside the C-terminal part of Avr2 other than cysteines did neither change the affinity of Avr2 mutants for Rcr3 nor their Cf-2-mediated HR-inducing activity.

As the Avr2 amino acid sequence and overall structure is distinct from other known protease inhibitors like E64 and cystatin, we studied the interaction between Rcr3 and wild type Avr2 in more detail. We performed inhibitor assays at four different concentrations of Avr2, constant Rcr3 concentrations and varying Bodipy FL-casein substrate concentrations. The data presented in a Lineweaver-Burk plot (Figure 4) showed that Avr2 is clearly not a competitive cysteine protease inhibitor but rather behaves as an uncompetitive inhibitor. This indicates that Avr2 most likely only binds the Rcr3-substrate complex outside the catalytic centre of Rcr3 and affects both V_m and K_m. We used Bodipy FL-casein as an artificial substrate to measure enzymatic of

Rcr3, as the native substrate in tomato for Rcr3 is not known. The K_i values of wild type and mutant Avr2 proteins towards Rcr3 are negatively correlated with Avr2-triggered Cf-2-mediated HR. Thus, the lower the affinity of Avr2 mutants for Rcr3, the lower the Cf-2-mediated HR-inducing activity. As Avr2 does not bind to the catalytic centre of Rcr3 this suggests that conformational changes of Rcr3 induced by binding of Avr2 trigger the Cf-2-mediated HR. This is in agreement with the observation that the *Lycopersicon esculentum* variant of Rcr3 ($Rcr3^{esc}$) can trigger a Cf-2-mediated HR in an Avr2-independent manner. $Rcr3^{esc}$ differs from $Rcr3^{pim}$ in only a few amino acids and these seem to be sufficient to cause a conformational change in the protein to trigger a Cf-2-mediated HR in an Avr2-independent manner.
A similar finding was reported for the affinity of Avr9 mutant peptides for a high-affinity binding site (HABS) and Avr9-triggered Cf-9-medited HR (Kooman-Gersmann et al., 1998). Alanine scanning mutants of Avr9 with the lowest affinity for the HABS showed the lowest Cf-9-mediated HR-inducing activity.
In conclusion, Avr2 is a potent cysteine protease inhibitor that is different from other known cysteine protease inhibitors like the phytocystatins (Rawlings et al., 2004). During evolution, Avr2 is required by *C. fulvum* to inhibit various extracellular plant cysteine proteases including Rcr3 and Pip1 to enable the pathogen to grow in the cysteine protease-rich environment of the apoplast of tomato. Indeed, it has been shown that Avr2 behaves as a genuine virulence factor of *C. fulvum* in the absence of the Cf-2 protein (Van Esse et al., 2008) and as an avirulence factor in the presence of the Cf-2 protein (Rooney et al., 2005).
Recently it was reported that *P. infestans*, a pathogen of tomato and potato, secretes two cystatin-like protease inhibitors, Epic1 and Epic2B, that both interact with, and inhibit Rcr3 but fail to trigger a Cf-2-mediated HR (Song et al., 2009). Also inhibition of Rcr3 by the irreversible inhibitor E-64 does not trigger a Cf-2-mediated HR (Rooney et al., 2005). This shows that inhibition of Rcr3 can be achieved by different cysteine protease inhibitors but that only the interaction with Avr2 triggers Cf-2-mediated HR, indicating that the conformational change in Rcr3 induced by Avr2 is different from those induced by cystatins, Epic1, Epic2B and E-64. These data also indicate that Avr2 is a unique cysteine protease inhibitor and that its interaction with Rcr3 results in a unique conformational change that is recognized by Cf-2. Interaction of the C-terminal tail of Avr2 with Rcr3 is most likely, but so far we were not able to identify the interaction site between Avr2 and Rcr3 by mass spectrometry.

Materials and methods

Plasmid constructs used for *Pichia pastoris* protein expression.

Plasmids for expression of Rcr3, wild type Avr2 and mutant Avr2 proteins in *Pichia pastoris*, were generated as described before (Rooney et al., 2005). Throughout this study the Rcr3 *pimpinellifolium* ($Rcr3^{pim}$) isoform was used.

Mutant Avr2 peptide production in *Pichia pastoris*

Pichia pastoris strain GS115 (Invitrogen) was transformed with plasmids encoding different mutant Avr2 peptides. The tags and the various mutants introduced in Avr2 are indicated in Figure 2 A and the different primers used to introduced the mutations are shown in Table I. The vector pPIC9 (Invitrogen) is used for secretion of the proteins in the culture medium. Transformants were analyzed by Tricine SDS-PAGE, and stained with Coomassie Brilliant Blue or analyzed on western blots, using antibodies (anti-Flag or anti-Avr2) against the tagged proteins. Transformants expressing significant amounts of the different Avr2 proteins were selected for fermentation. Fermentation was performed as previously described (Rooney et al., 2005). The tagged proteins were subsequently purified on a Ni-NTA column and fractions containing pure protein were pooled and dialyzed against water. For mass spectrometric analysis of Avr2 the His-Flag tag was removed by treatment with enterokinase. Protein concentrations were determined by BCA protein assay (Pierce artno: 23225). Purified wild type and Avr2 mutants were tested for their Rcr3 inhibitory activity and their potential to trigger a Cf-2-mediated HR. Plants were monitored for HR development 3 to 5 days post injection.

Determination of apparent equilibrium dissociation constants.

For a selection of Avr2 mutant proteins which showed an altered Cf-2-mediated HR, their apparent equilibrium dissociation constants (K_i) for Rcr3 was determined by titration of the enzymes using curve fitting software as described (Annadana et al., 2003). For determination of the K_i, different concentrations of each Avr2 mutant protein were added to Rcr3 and the remaining Rcr3 enzyme activity was measured by using Bodipy FL-casein as a substrate in presence of 10 mM L-cysteine, 50 mM NaAcetate, pH 5.0 (Annadana et al., 2003). The fluorescence was measured in a fluorescence microplate reader with standard fluorescein excitation filters at 485 ± 12.5 nm and emission at 530 ± 15 nm. All K_i measurements were performed in triplicate. Fluorescence values were fitted to the K_i modeling (Beekwilder et al., 2000).

Lineweaver-Burk plots

The V_{max} and K_m of Rcr3 were measured indirectly, by determining Rcr3 activity under various substrate and Avr2 concentrations. The effects of different Avr2 concentrations on Rcr3 activity were visualized using Lineweaver-Burk plots allowing to draw conclusions on the mode of inhibition (competitive, un-competitive, non-competitive or mixed), where competitive inhibitors affect K_m, non-competitive inhibitors affect V_{max} and un-competitive inhibitors affect both V_{max} and K_m, and mixed inhibitors show intermediate patterns. For each Avr2 concentration a fixed Rcr3 concentration was used and the Bodipy FL-casein substrate was varied. All tests were performed in 10 mM L-cysteine, 50mM NaAcetate, pH 5.0. All samples were measured 0, 2, 4, 6 and 24 hours after incubation at room temperature.

Figure and figure legends.

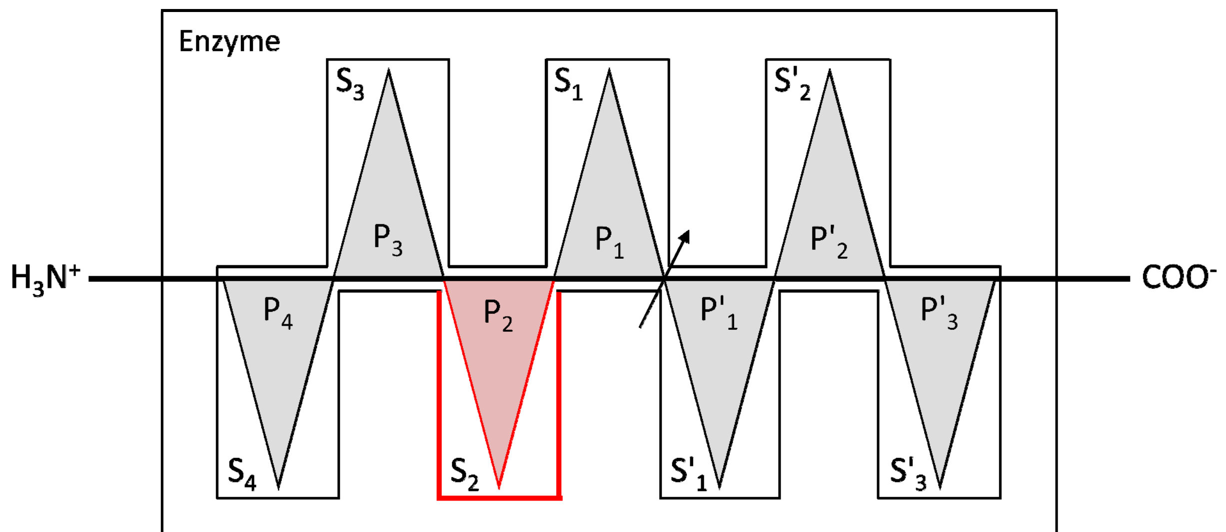

Figure 1. Schematic representation of the active centre of papain-like cysteine proteases (PLCPs) showing seven enzyme subsites S_1 to S_4 and S'_1 to S'_3, each capable of accommodating a side-chain of a corresponding amino acid residue of a peptide substrate or peptide inhibitor, P_1 to P_4 and P'_1 to P'_3. The arrow indicates the peptide bond between P_1 and S'_1 that is hydrolyzed during catalysis. S_2 determines specificity of PLCPs (according to Turk et al., 1998).

1 10 20 30 40 50 58

HHHHHHDYKDDDDK A K K L P G C D K D P C K V K E K S G K Y K L K I G A K C S A T C D G K L T R G G T C E N V Q G N H L C **C F G L C G**

Figure 2A. Amino acid sequence of wild type Avr2 protein (residue 1-58) including the His (HHHHHH) and the Flag (DYKDDDDK) tags at the N-terminus. The enterokinase site is indicated by a triangle.

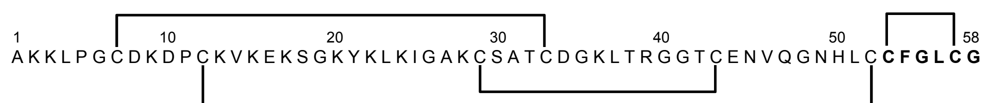

Figure 2B. The disulfide bond pattern of wild type Avr2 as determined by disulfide mapping combined with MALDI-TOF MS analysis. The disulfide bonds between the cysteine residues are indicated as solid lines.

Figure 2C. Amino acid substitutions introduced in the wild type His-Flag tagged Avr2 protein. Underlined amino acid residues have been substituted by alanine. The constructs encoding mutant versions of Avr2 were produced in *Pichia pastoris*. One mutant Avr2 was produced lacking the six C-terminal amino acid residues (indicated in bold) leading to a 52-amino acid mutant Avr2 protein. For His-Flag tag see legends of Figure 2A.

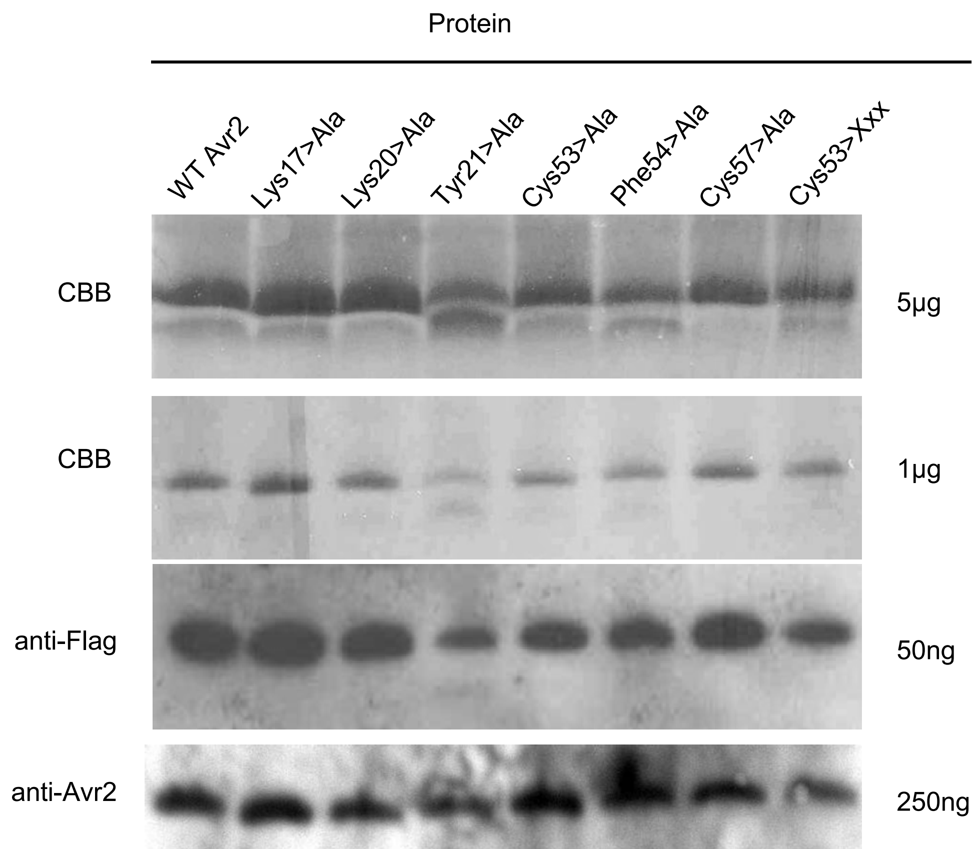

Figure 3. SDS-PAGE of wild type His-Flag-tagged wild type Avr2 and mutant Avr2 proteins containing various amino acid substitutions or a C-terminal deletion of six amino acids (Cys 53>Xxx).

Equal amounts of protein (as indicated) were loaded on the gel; proteins were stained with Coomassie Brilliant Blue and identified by western blot analysis using anti-Flag and anti-Avr2 antibodies.

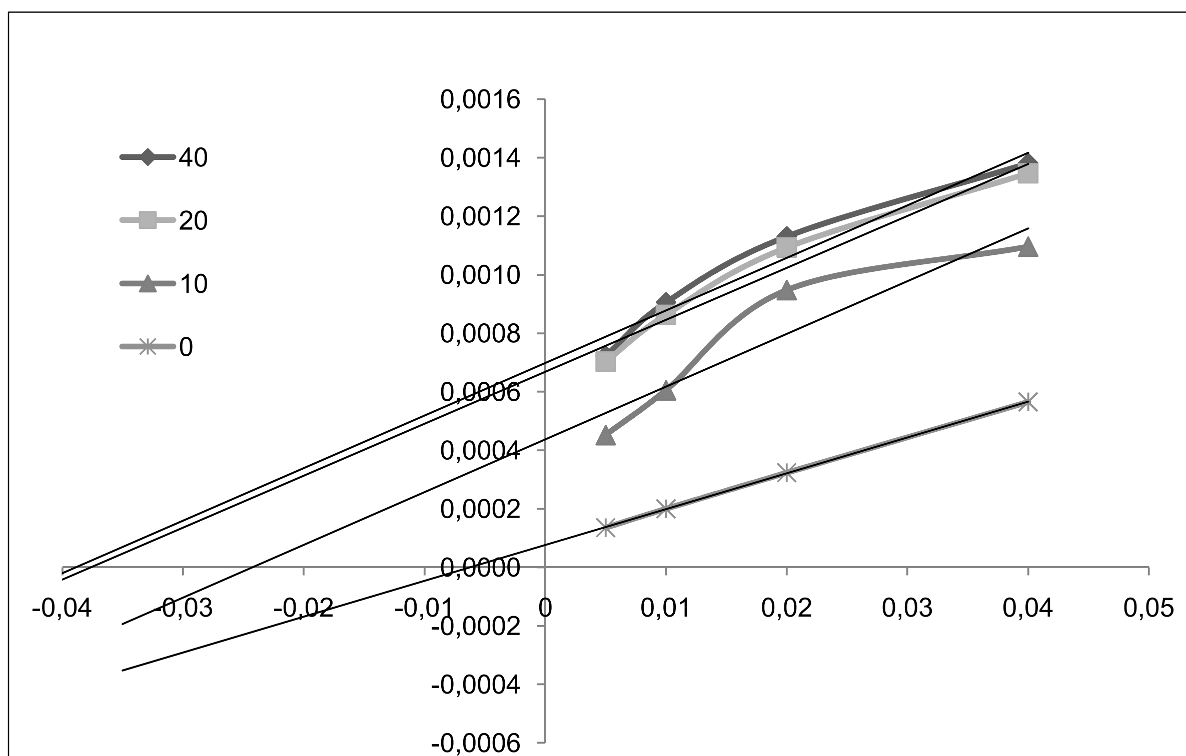

Figure 4. Lineweaver–Burk plot analysis.
Rcr3 activity was measured at four different concentrations of Avr2 (0, 10, 20 and 40 µM) at four different concentrations 230, 460, 920 and 1840 nM of the substrate Bodipy FL-casein for 24hrs at RT. The observed inhibition pattern is most similar to un-competitive inhibition.

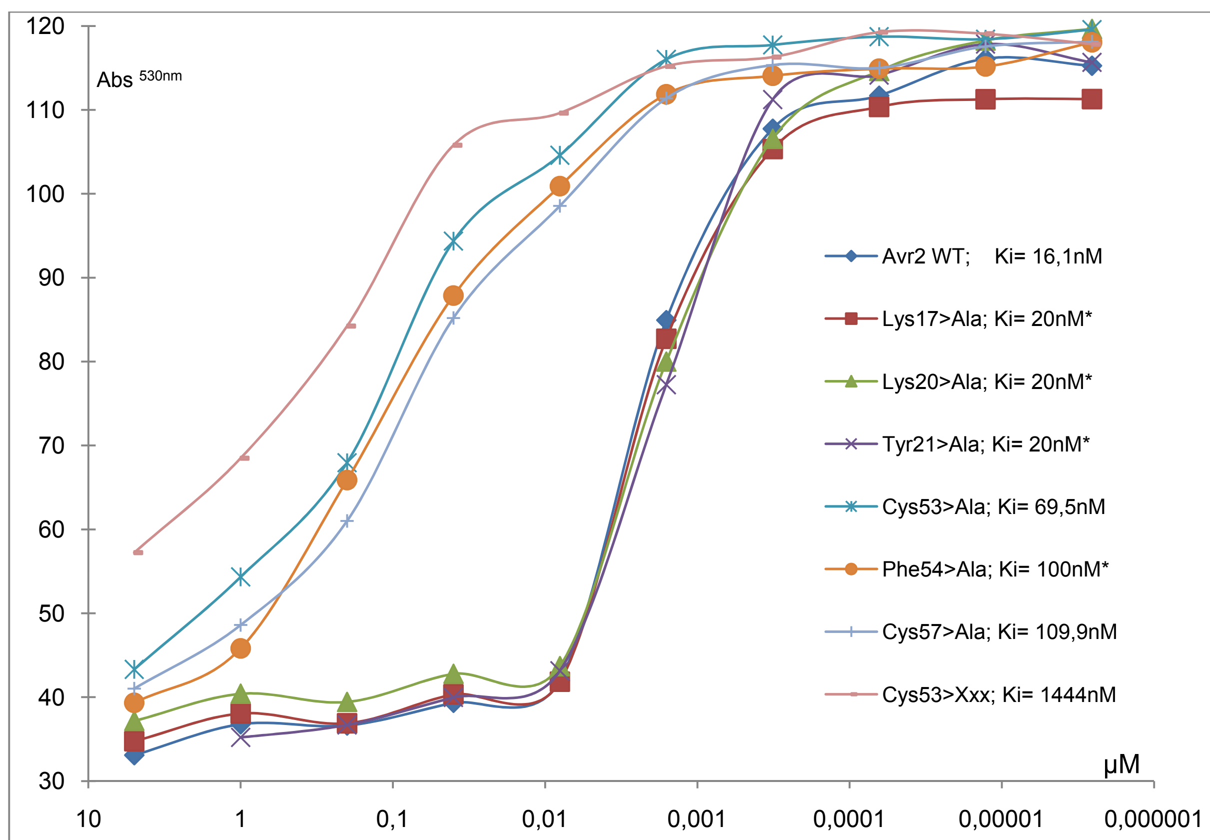

Figure 5. The affinity of wild type and mutant Avr2 proteins for Rcr3.
Wild type or mutant Avr2 proteins were incubated at different concentrations with constant concentrations of Rcr3 and the substrate Bodipy FL-casein. The rest activity of Rcr3 was measured and the K_i values (indicated in the insert) were determined as described in Materials and Methods.

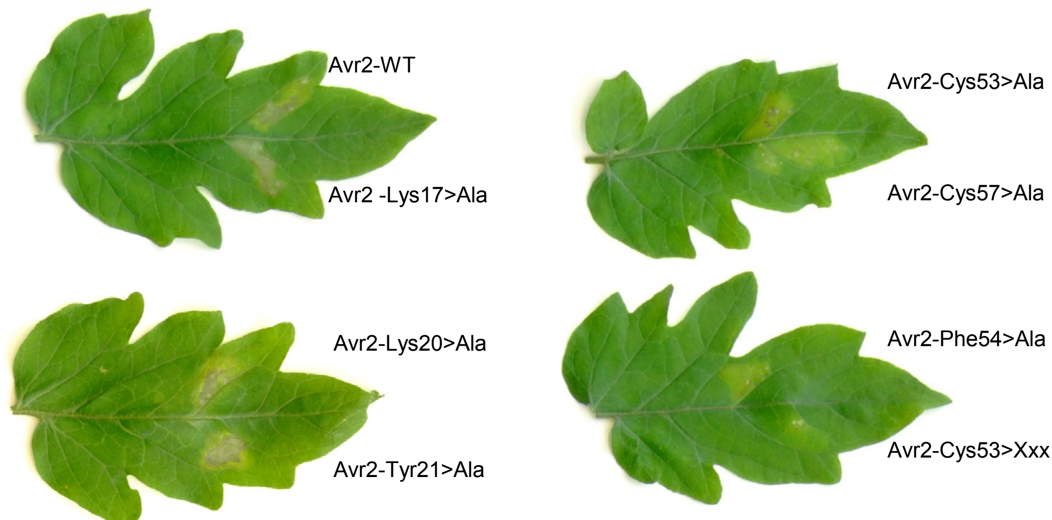

Figure 6. **Cf-2-mediated HR activities triggered by wild type and mutant Avr2 proteins produced in *P. pastoris.***

Photograph was taken three days post injection of 100 µl of an aqueous solution containing 2µM of wild type or mutant Avr2 protein as indicated in photograph. Note that only mutations in the six C-terminal part of Avr2 did significantly affect the Cf-2-mediated HR activities. Deletion of the six C-terminal amino acid residues of Avr2 abolished Cf-2-mediated HR activity nearly completely. (compare also Cf-2-mediated HR-inducing activities with K_i values presented in Figure 5).

Table 1. **Primers used in this study**.
Primer codes should be read as follows; A2 stands for Avr2, next four characters indicate the amino acid residue and its position in the Avr2 protein sequence to be substituted by alanine, except for A2C53XF and A2C53XR which leads to a protein with a C-terminal six amino acid deletion.
F and R stand for forward and reverse primer sequence.

Code	Sequence
A2K17AF	GTGAAGGAG**GC**GTCTGGAAAG
A2K17AR	CTTTCCAGAC**GC**CTCCTTCAC
A2K20AF	GGAGAAGTCTGGAGCGTATAAATTG
A2K20AR	CAATTTATACGCTCCAGACTTCTCC
A2Y21AF	GGAGAAGTCTGGAAAGGCTAAATTGAAG
A2Y21AR	CTTCAATTTAGCCTTTCCAGACTTCTCC
A2C29AF	GATTGGTGCTAAA**GC**CTCGGCGACATGTG
A2C29AR	CACATGTCGCCGAG**GC**TTTAGCACCAATC
A2C33AF	GGTGCTAAATGCTCGGCGACA**GC**TGACGGG
A2C33AR	CCCGTCA**GC**TGTCGCCGAGCATTTAGCACC
A2C52AR	GGGTAATCACCTT**GC**CTGTTTTGGTCTTTGCGG
A2C52AF	CCGCAAAGACCAAAACAG**GC**AAGGTGATTACCC
A2C53AR	GGGTAATCACCTTTGC**GC**TTTTGGTCTTTGCGG
A2C53AF	CCGCAAAGACCAAAA**GC**GCAAAGGTGATTACCC
A2F54AF	CACCTTTGCTGTGCTGGTCTTTGC
A2F54AR	GCAAAGACCAGCACAGCAAAGGTG
A2C57AR	CCTTTGCTGTTTTGGTCTT**GC**CGGTTGATGATACG
A2C57AF	CGTATCATCAACCG**GC**AAGACCAAAACAGCAAAGG
A2C53XF	GGGTAATCACCTTTGCTGATAGTGAGAATTCCG
A2C53XR	CGGAATTCTCACTATCAGCAAAGGTGATTACCC

CHAPTER 5

General Discussion: The arms race between cysteine proteases and cysteine protease inhibitors in microbial pathogen-plant host interactions

Early studies on the interactions between tomato (*Solanum lycopersicum*) and both the fungal pathogen *Cladosporium fulvum* and the oomycete pathogen *Phytophthora infestans* have revealed that high levels of pathogenesis-related (PR) proteins accumulate in the apoplast of the host upon infection (Kombrink et al., 1990; Joosten and De Wit, 1988).
Some of these PR proteins display enzymatic activity acting directly on these pathogens, for instance by degrading particular cell wall components (Van Loon et al., 2006; Ferreira et al., 2007), whereas several secreted pathogen effectors function as inhibitors of such host enzymes. As a result, selection pressure on host enzymes to evade inhibition by pathogen effectors and on inhibitory pathogen effectors to adapt to the altered host enzymes occurs (Misas-Villamil and van der Hoorn, 2008). The tomato-*C. fulvum* pathosystem is an ideal model to study the biochemistry of plant-pathogen interactions, since the interaction is confined to the apoplast which is the only compartment of the host that is colonized by the fungus.
Although fungi and oomycetes cause major economic damage, bacteria are still the best characterized plant pathogens mainly due to the fact that molecular tools such as transposon mutagenesis, transfection, transformation with phages and plasmids could be used for bacteria but not for fungi and oomycetes that could only be transformed successfully by stable integration of DNA in the late eighties of last century. Besides, the genomes of bacteria are on average two orders of magnitude smaller than those of fungi and oomycetes (Hennecke and Verma, 1990). Bacterial pathogens operate in the intercellular space of the host. Many bacterial whole genome DNA sequences are now publicly available, including those of *P. syringae*, *P. phaseolicola*, *Ralstonia solanacearum*, *Xanthomonas campestris*, *X. oryzae*, *Xylella fastidiosa*, and their effector catalogues have been predicted. To date, most information is available on type three secreted effectors from bacterial plant pathogens. A large number of these effector molecules show enzymatic activity, like phosphotases, ligases, transferases and proteases. Remarkably all these prokaryotic proteases are characterized as cysteine proteases as reviewed by Göhre and Robatzek (2008).
To date, much progress has been made in molecular research on fungi and Oomycetes, and most molecular techniques such as transformation, knocking out or knocking down genes have become a daily routine. Also genome sequences of many pathogenic fungi and Oomycetes have become available including the genes encoding effectors. Many eukaryotic plant pathogen effectors show an indirect or direct inhibitory effect on plant defense enzymes (Kamoun, 2008; Stergiopoulos and de Wit, 2009).
In this chapter, I will focus on the cysteine proteases and cysteine protease inhibitors as weapons employed in attack and defense, both by microbial pathogens and host plants.

1. Proteases

Proteases, also known as proteinases, peptidases or proteolytic enzymes, constitute a large group of enzymes which catalyze the hydrolysis of peptide bonds that link amino acids in the polypeptide chain of a protein with the participation of a water molecule, a process that is also known as proteolysis (Rawlings and Barrett, 2004).

Proteases are divided into exopeptidases that remove amino acids from the N-terminus (aminopeptidases) or C-terminus of a protein (carboxypeptidase), and endopeptidases that can hydrolyze peptide bonds of non-terminal amino acids (i.e. within the polypeptide chain). Endopeptidases are usually highly specific for certain amino acids and hydrolyze specific peptide bonds, often giving rise to peptides with specific biological functions (limited proteolysis). However, proteolysis can also result into complete breakdown of a protein into its constituting amino acids (unlimited proteolysis).

All organisms possess protease genes, which comprise on average up to 5% of the genes in a genome. Proteases can be involved in a multitude of physiological processes, ranging from simple digestion of proteins to regulation of signaling cascades in the cytoplasm, and are found in organelles like mitochondria, lysosomes, peroxisomes, vacuoles and the endoplasmic reticulum (ER). Proteolysis represents one of the fastest molecular "start" and "stop" mechanisms in the physiology of an organism. In general, signaling pathways initiated by proteases are irreversible, although exceptions have been described (Turk, 2006).

2. Classification of proteases.

The mechanism used by proteases to cleave peptide bonds involves a nucleophylic attack on the peptide carbonyl group, as proteases catalyze protein hydrolysis after covalent or non-covalent interactions with their substrates (Turk, 2006). Proteases are classified into five catalytic types: (i) cysteine proteases, (ii) serine proteases, (iii) threonine proteases, (iv) aspartic proteases and (v) metalloproteases (http://merops.sanger.ac.uk; Rawlings et al., 2010).

Covalent catalysis is performed by cysteine, serine and threonine proteases that have a Cys, Ser or Thr amino acid, respectively, in their catalytic centre which becomes the nucleophile and requires a His residue as a base. Metallo and aspartic proteases follow a non-covalent catalytic mechanism with an activated water molecule as the nucleophile. A zinc molecule serves as base for metalloproteases, whereas an Asp or a Glu residue serves as acid for the aspartic proteases (Figure 1).

Proteases of all five catalytic types are represented in all plant genomes that have been investigated thus far. *Arabidopsis thaliana* contains over 800 protease genes and rice (*Oryza sativa*) nearly 700 (van der Hoorn, 2008). Serine, cysteine, and asparitic proteases are the most widely represented in plants, of which the serine proteases that belong to the subtilisin-like family (S8A) are the most widespread.

Plant proteases have been implicated in diverse physiological processes such as seed germination, growth, xylogenesis, leaf and flower senescence, fruit ripening, seed development and cell death. Proteases also play a role in responses of plants to abiotic stresses including water and nutrient deprivation, extreme temperatures and osmotic shock. Furthermore, proteases play a role in responses to biotic stresses, where some proteases act directly against plant pathogens, whereas others are involved in perception of pathogens or in activation of defense signaling cascades that may culminate in the hypersensitive response (HR).

2.1 Cysteine proteases

In this paragraph, I will focus on the plant cysteine proteases involved in, or associated with, programmed cell death (PCD) or the HR. Plant cysteine proteases are divided into 15 families of 5 clans (http://merops.sanger.ac.uk; Rawlings et al., 2010). Clans CA and CE contain proteases with a papain-like fold, whereas CD proteases have a caspase-like fold. Many of these cysteine proteases play a role in PCD.

2.1.1 Caspase-like proteases of the CD-clan

PCD is a phenomenon that plays an important role in growth, development and defense of eukaryotic organisms including plants, and proteases have shown to play an important role in this process (Turk et al., 2007). PCD is very well studied in animal model organisms such as the fruit fly *Drosophila melanogaster* and the nematode *Ceanorhabditis elegans.* A distinct group of cysteine proteases, the cysteine-dependent aspartate-specific proteases or caspases, play a central role in the execution phase of PCD in mammals (Leist and Jäättelä, 2001), but are also involved in non-apoptotic processes, like inflammation, cell proliferation and cell differentiation (Lamkanfi et al., 2007; Overholtzer et al., 2007).

Mammalian caspases belong to the CD clan, the C14 family of cysteine proteases and have specific preference for the Asp residue at the P1 position of peptide bonds in the substrate (Figure 2) (http://merops.sanger.ac.uk; Rawlings et al., 2010).
Animal caspases are constitutively produced and are synthesized as inactive pre-pro-enzymes (Martin and Green, 1995; Martins et al., 1997). They can be divided into two types based on their overall structure and mode of activation, with type-I caspases functioning as effectors and type-II caspases as initiators. Initiator caspases have an N-terminal prodomain that is needed for recruitment to protein complexes, known as apoptosomes, that function as activation platforms (Riedl and Salvesen, 2007). They are activated by conformational changes after oligomerization, for which proteolytic cleavage is not required (Fuentes-Prior and Salvesen, 2004). In turn, they activate the effector caspases by the proteolytic separation of the large (p20) and small (p10) subunits and finally form active

(p20)2(p10)2 heterotetramers. Effector caspases are known to cleave numerous substrates and cause typical morphological features of apoptosis (Kumar, 2007; Timmer and Salvesen, 2007).

Mammalian caspases (Clan CD, family C14) possess three characteristic features: they contain (i) a caspase fold, (ii) are involved in PCD and (iii) show caspase-like activities. Plant proteases with these features are found in two cysteine protease families: (i) vacuolar processing enzymes (VPEs; Clan CD: family C13) and (ii) metacaspases (Clan CD: family C14; Chen et al., 1998) (Figure 3). Metacaspases and mammalian caspases share similar functions but have a different substrate preference with an Arg residue at the P1 position of the substrate for metacaspases, instead of an Asp residue at this position for mammalian caspases. Separation of the group of the mammalian caspases from the metacaspases is supported by phylogenetic analyses of clan CD peptidases (Koonin and Aravind, 2002), which indicates that metacaspases are clearly distinct from mammalian caspases (Vercammen, et al., 2007).

In plants, PCD is an essential phenomenon that occurs during terminal differentiation of the vascular tracheary elements, leaf and flower senescence, elimination of reproductive organs in unisexual flowers, pollen rejection in the self-incompatibility response, fruit dehiscence, or pod shattering, and caspase activity is detected throughout the life cycle of plants, from seed germination until seed ripening (Fukuda, 2000; van Doorn and Woltering, 2005). PCD in plants covers at least three major cytological mechanisms (Jones, et al., 2001; Kuriyama and Fukuda, 2002). During senescence, initially, the chloroplasts are degraded followed by disruption of the vacuoles and the nucleus (Thomas et al., 2003). The second mechanism involves disruption and collapse of the large central vacuole, release of nucleases and proteases, acidification of the cytoplasm, and rapid degradation of nucleic acids and proteins (Obara et al., 2001; Kuriyama and Fukuda, 2002). Thirdly, in cells that undergo apoptosis-like PCD, the nucleus is degraded first, after which chromatin condenses and DNA undergoes laddering (Fukuda, 2000; Ondzighi, et al., 2008).

Resistant plants respond with an HR to infection by biotrophic pathogens in order to prevent further pathogen spread (Jones and Dangl, 2006; de Wit, 2007). In dying plant cells apoptotic features such as chromatin condensation, nuclear rupture, internucleosomal DNA cleavage, cell shrinkage, and the formation of apoptotic bodies have been observed (van Doorn and Woltering, 2005). Studies with synthetic caspase-specific inhibitors such as Ac-YVAD-CHO and Ac-DEVD-CHO and caspase substrates confirmed the existence of caspase-like activities in plants and their requirement for completion of PCD (del Pozo and Lam, 1998; Watanabe and Lam, 2004; Woltering, 2004).

The whole genome sequences of *A. thaliana* and rice do not show direct orthologs of animal caspase sequences, and therefore plant caspase activities are usually referred to as caspase-like or DEVDase in case they cleave the protein recognition

site Asp-Glu-Val-Asp. Today, at least eight caspase-like activities in plant extracts have been reported using caspase substrates, and some occur during tobacco mosaic virus (TMV)-induced cell death in *Nicothiana benthamiana* and also developmental cell death (Xu and Zhang, 2009).

Initially, caspase-like activities were assigned to the vacuolar processing enzymes (VPEs) and the metacaspases (Woltering et al., 2002). Both show some sequence and tertiary structure homology with animal caspases and have been suggested to regulate PCD in plants as true caspase orthologs (Aravind and Koonin, 2002; Hatsugai et al., 2004; Rojo et al., 2004; Suarez et al., 2004; Sanmartin, 2005). However, in oat (*Avena sativa*) a serine protease, referred to as saspase, has been identified that also displays caspase-like activity and which may control PCD in association with victorin toxin-induced cell death (Coffeen and Wolpert, 2004). Saspase cleaves caspase-8 and some caspase-6 synthetic substrates. Nevertheless, a major challenge is still to identify the plant proteases that are responsible for the caspase-like activities and to understand their relationship with animal caspases. It is now more than 10 years ago since the first report of caspase activity in plants (del Pozo and Lam, 1998) and several scenarios to explain why plants have caspase-like activities without animal-like or true caspases have been proposed.

2.1.2 Vacuolar processing enzymes

Vacuolar processing enzymes (VPEs) belong to the CD clan and C13 family of cysteine proteases and show specificity in the P1 position for Asp. VPEs and mammalian caspase-1 share the same substrate specificity (Hatsugai et al. 2006). VPEs play a role in various protein maturation steps in seed storage vacuoles, but are also synthesized in vegetative plant tissues and hydrolyze caspase-specific substrates (Shimada et al., 2003; Gruis et al., 2004). Furthermore, VPEs are localized in protease precursor vesicles (PPVs) and vacuoles, organelles closely associated with PCD.

The *A .thaliana* genome harbors four genes encoding VPEs. Two of them are VPEα and VPEγ, and both are localized in lytic vacuoles of vegetative organs and are involved in PCD induced by various stress factors and during ageing (Kinoshita et al. 1999). Moreover, *in vivo* studies have provided evidence for the involvement of *A. thaliana* VPEγ in protein degradation during senescence and in disease resistance and cell death progression (Rojo et al., 2003; 2004). The predicted three-dimensional structure of *A. thaliana* VPEγ shows resemblance of its active site with human caspase-8 (Rojo et al., 2003).

A. thaliana plants infected by an avirulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 show increased caspase activity and initiation of PCD. *VPEγ* mutants have compromised caspase activity and are more susceptible to *P. syringae* and to turnip mosaic virus (TuMV; Rojo et al., 2004). The latter pathogen does not induce a

classical PCD but decreases the viability of infected cells. Apparently, VPEs negatively regulate the growth of these pathogens by promoting cell death. This contrasts to studies where *VPEγ* mutants were reported to be more susceptible to *Botrytis cinerea*, a necrotrophic pathogen that is expected to benefit from PCD (Govrin and Levine, 2000; Dickman et al., 2001). These data indicate a key role for VPEs in PCD in interactions of *A. thaliana* with pathogens (Sanmartin, 2005).

2.1.3 Metacaspases

The *A. thaliana* genome contains nine predicted metacaspase genes of which three belong to the type-I class (*AtMC 1-3*) and six to the type II class (*AtMC4-9*). Type-I metacaspases possess a predicted N-terminal prodomain with a zinc finger motif followed by a proline-rich region (*AtMC2* and *AtMC3*) or by a glutamine- and proline-rich region (*AtMC1*) and a C-terminal caspase-like domain. *AtMC4-9* lack the obvious prodomain (Watanabe and Lam, 2004; Uren, et al., 2000).

Transcripts of *AtMC1-3*, *-5* and *-6* are rapidly up-regulated upon infection of leaves with bacterial pathogens. Similarly, *LeMCA1*, encoding a type II metacaspase of tomato, was upregulated in leaves infected by *B. cinerea* (Hoeberichts, 2003). These data suggests the possible involvement of metacaspases in plant defense.

In Norway spruce (*Picea abies*), a type II metacaspase gene, *mcII-Pa*, was shown to be involved in somatic embryogenesis (Suarez, et al., 2004). Previously, it has been reported that activation of (a) protease(s) cleaving the caspase substrate Val-Glu-Ile-Asp (VEIDase) is essential for PCD and embryogenesis in Norway spruce (Bozhokov, et al., 2004). The metacaspase gene *mcII-Pa* may encode a functional homolog of animal caspase-6 (VEIDase) and silencing of *mcII-Pa* strongly reduced VEIDase activity, suppressed PCD in the embryos and blocked suspensor differentiation (Suarez, et al., 2004). However, it has subsequently been shown that mcII-Pa does not contain VEIDase activity, as active mcII-Pa does not possess aspartic acid-specific proteolytic activity but prefers Arg-containing substrates (Bozhokov, 2005). This suggests that the caspase activity is not caused by mcII-Pa activity, but by enzymes activated by mcII-Pa. Nuclei isolated from PCD-deficient cell lines are disintegrated by adding mcII-Pa protein and this disintegration can be inhibited by a mcII-Pa inhibitor and is absent when a catalytic mutant of mcII-Pa is added instead. It is hypothesized that cytoplasmic metacaspases participate in PCD by degrading the nuclear envelope, which leads to nuclear degradation (Bozhokov et al., 2005).

Although no functional homologs of animal caspases have been identified in plant genomes, data are accumulating that suggest the existence of caspase-like activity in plants which is functional direct or indirect in various types of plant PCD.

2.2 Papain-like cysteine proteases

Papain is the type protease of the papain family and is found in latex of papaya (*Carica papaya*) plants and acts in defense against herbivore insects (Konno et al., 2004).

Papain-like cysteine proteases (PLCPs) originate from a common evolutionary ancestor and a number of them are strongly expressed during senescence (Guo et al., 2004).Treatment with specific PLCP inhibitors, such as E64, or over-expression of endogenous PLCP inhibitors, such as phytocystatins blocks PCD induced by various stimuli, including hydrogen peroxide (H_2O_2), nitric oxide (NO) and avirulent bacterial strains (Solomon et al., 1999; Belenghi et al., 2003; Coffeen & Wolpert, 2004). Cumulatively, these data suggest that PLCPs play a role in PCD.

2.2.1 Cathepsin B

In addition to caspases, another group of lysosomal cysteine and aspartate proteases is involved in PCD in animals; the cathepsins (Leist and Jäättelä, 2001; Turk and Stoka, 2007; Vasiljeva and Turk, 2007). Mammalian cathepsin B (CathB) has been shown to activate specific caspases by cleaving their prodomains (Vancompernolle et al., 1998) and is able to execute caspase-independent apoptosis upon tumor necrosis factor (TNF) receptor activation (Foghsgaard et al., 2001). CathB knock-out mice mutants show reduced TNF-triggered apoptosis (Guicciardi et al., 2001; Reinheckel et al., 2001). Cathepsins can cleave Bid, a pro-apoptotic factor (Cirman et al., 2004; Blomgran et al., 2007) or degrade the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL (Turk and Stoka, 2007).

The *Nicotiana benthamiana* CathB-like (NbCathB) protease is activated upon secretion in the apoplast in absence of pathogen challenge. Virus-induced gene silencing (VIGS) of *NbCathB* suppressed the HR that is triggered by co-expression of the *Phytophthora infestans* effector *Avr3a* and its cognate potato *R* gene, but did not suppress the HR in tomato following recognition of *C. fulvum* Avr4 by tomato Cf-4. Furthermore, VIGS of *NbCathB* enhanced susceptibility to the nonhost bacterial pathogens *Erwinia amylovora* and *P. syringae* pv. *tomato* DC3000 (Gilroy et al., 2007). It was subsequently demonstrated that three *A. thaliana CathB* homologs are redundantly required for basal resistance to Pst DC3000. They are not required for *R* gene-mediated resistance to avirulent strains of Pst DC3000 expressing *AvrB* or *AvrRps4*, but are required for the activation of HR upon AvrB recognition (McLellan et al., 2009). In addition, it was demonstrated that these proteases are required for activation of the senescence-associated marker gene (SAG12), suggesting that CathB is a regulator of PCD both in senescence and basal defense (McLellan et al., 2009).

2.2.2 Rcr3

By mutational analysis of *Cf-2* tomato plants *Rcr3* was identified as a gene required for *C. fulvum* resistance. *Rcr3* is only required for *Cf-2* function but not for functionality of other known *Cf* resistance genes (Dixon et al., 1998). Rcr3 appeared to be an apoplastic cysteine protease and is 43% identical to papain. *Rcr3* is upregulated in adult plants, during senescence and during infection by *C. fulvum*. Similar to other typical *PR* genes, *Rcr3* expression is induced more rapidly in incompatible interactions of tomato plants containing *Cf2* or *Cf9*, when compared to compatible interactions of tomato plants (Krüger et al., 2002).

By EMS mutagenesis four *rcr3* mutant *Cf-2* plants were acquired, three of which will be discussed here. The *rcr3-1* mutant does not fully compromise Cf-2 function, whereas the *rcr3-2* and *rcr3-3* mutants result in a complete loss of Avr2-triggered Cf-2-mediated resistance. The *rcr3-1* mutant contains a point mutation in the *Rcr3* gene, Cys 151>Ser mutation), and is most likely affected in its 3-D structure since this cysteine is involved in a disulphide bridge. The Rcr3 wild type protein contains 8 Cys residues, in contrast to the rcr3-1 mutant. Possibly, the Cys153 will be involved in a disulphide bond instead of the Cys151, which may result in an altered 3-D structure. This protein could display a reduced enzyme activity, although Avr2 is still able to interact with the mutant protein and this complex is still recognized by Cf-2.

The *rcr3-2* allele contains a point mutation that results in a Gly314>Val substitution.. Gly 314 belongs to the Try-Gly motif that is highly conserved in PLCPs and which is in close proximity to the Asn residue of the catalytic triade (Cys-His-Asn). It is conceivable that the rcr3-2 mutant protein is stable and similarly folded as the Rcr3 wild type protein, although it is no longer an active enzyme. These data show that enzymatic activity of Rcr3 is required for the Avr2- triggered Cf-2-mediated HR.

The *rcr3-3* allele encodes a premature stop codon at DNA sequence position 221, resulting in a truncated non-functional protein.

It was proposed that Rcr3 would solely act as a decoy to bind Avr2 and thus recognize the pathogen, leading to Cf2-mediated HR (Shabab et al., 2008), whereas Pip1 (*Phytophthora* inhibited protease 1) would be the real virulence target of Avr2. In this so-called decoy model it is hypothesized that Rcr3 which is guarded by Cf-2 is solely involved in Avr2 perception, and thus binding of Avr2 to Rcr3 in absence of Cf-2 would not contribute to pathogen virulence (Van der Hoorn and Kamoun, 2008). However, a recent study has shown that the tomato *Cf-2/rcr3* mutant confers increased susceptibility to *P. infestans*, which indicates an active role for Rcr3 in plant defense, which argues against a role of Rcr3 as a decoy (Song et. al., 2009). Furthermore, Rcr3 has a putative function in senescence as described by Krüger et al. (2002). And finally, *Cf-2/rcr3* plants show a delay in growth when compared to *Cf-2/Rcr3* tomato, which indicates that Rcr3 contributes to plant growth.

During senescence or pathogen attack the expression of *Rcr3* is upregulated. Similar transcriptional regulation is observed for the gene encoding the CathB-like protease,

which may function in downstream signaling (McLellan et al., 2009). Interestingly, also the extracellular CDR1 aspartic protease of *A. thaliana* has a role in disease signaling (Xia et al., 2004). Comparable to CathB-like and CDR1, Rcr3 could act indirectly in defense as a messenger in signaling to downstream defense mechanisms or catalytically process an endogenous substrate into an activate enzyme or molecule that acts directly towards the pathogen. Alternatively, these products could act in PCD signaling. Interestingly PR-1 expression is induced upon Avr2 protein injection in *Cf-2/Rcr3^{pim}* tomato but not in *Cf-2/rcr3^{pim}* (Song et al., 2009). The majority of the PR proteins in the apoplast are enzymes that act in basal defense. Uncontrolled or unregulated enzymes are harmful and therefore it is desirable to keep such enzymes inactivated until their activity is required. The Rcr3 protein level is nearly undetectable in unchallenged tomato plants (Chapter 2), which is in contrast to other apoplastic PR proteins. All these data suggests that Rcr3 might enzymatically activate PR proteins or itself, and its substrate might be involved in disease signaling. An example of such a process is Aleurain, a PLCP which is targeted to the lytic vacuole where it becomes active after proteolytic processing (Holwerda et al., 1990; Paris et al., 1996).

The Rcr3^{esc} variant shows autonecrosis in combination with *Cf-2* expression. This phenotype is observed after the onset of flowering, and follows a time pattern that is similar to the *Rcr3* expression in *Cf-2* plants. This Rcr3^{esc}-Cf-2-dependent autonecrosis can be suppressed by the Rcr3^{pim} allele. The Rcr3^{esc} protein differs from Rcr3^{pim} in one amino acid deletion and six amino acid changes, which may mimic the conformational change, imposed on Rcr3^{pim} upon Avr2 binding, and weakly activates *Cf-2*-dependent HR in the absence of Avr2. Modification of an enzyme by an inhibitor is a result of either un- or noncompetitive inhibition. Inhibition of Rcr3 by Avr2 is clearly not competitive but most likely un- and non-competitive, which is in agreement with a conformational change imposed by Avr2 on Rcr3 (Chapter 4). Another scenario might be that the Rcr3^{esc} variant is not able to process the endogenous substrate as efficiently as Rcr3^{pim} and the substrate-Rcr3^{esc} complex is recognized by Cf-2 leading to the autonecrosis. The uncompetitive Avr2 inhibition of Rcr3 implies formation of a substrate-Rcr3-Avr2 complex which is recognized by the Cf-2 protein. This could point towards the specificity of this interaction which leads to the Cf-2-mediated HR which does not occur in the Avr2-Pip1 interaction. Another peculiarity is observed in *Nicotiana tabacum* expressing Cf-2, where no HR occurs upon Avr2 and Rcr3 protein injection although in *N. tabacum* expressing Cf-4 or Cf-9 an HR is visible 3 days after injection of Avr9 or Avr4 protein, respectively. In contrast, in *N. benthamiana* transiently co-expressing *Cf-2, Avr2* and *Rcr3* an HR does occur, and autonecrosis is observed when transiently co-expressing *Cf-2* and *Rcr3^{esc}*. This could point towards the presence of an Rcr3 substrate that is present in *N. benthamiana* but absent in *N. tabacum*.

Both the modification of Rcr3 by interaction with Avr2 and the formation of a substrate-Rcr3-Avr2 complex could initiate a Cf-2 dependent HR. To date, however, the substrate of Rcr3 has not yet been identified.

2.2.3 Pip1

In a biochemical analysis, tomato Pip1 (*Phytophthora* inhibited protease 1) was identified and found to interact with the *P. infestans* secreted apoplastic effector EpiC1 (extracellular proteinase inhibitor C1) and EpiC2B (extracellular proteinase inhibitor C2B) in a similar manner as cystatin-like protease inhibitors. Pip1 is closely related to Rcr3, and like the *Rcr3* gene also the *Pip1* gene follows the expression pattern of a typical *PR* gene (Tian et al 2000). Pip1 is more abundant in the apoplast than Rcr3.

It was recently demonstrated that, similar to Avr2, also EpiC1 and EpiC2B bind and inhibit Rcr3. However, in contrast to Avr2, EpiC1 and EpiC2B do not trigger HR on *Cf-2* tomato. Nevertheless, the *rcr3-3* mutant of tomato that carries a premature stop codon in the *Rcr3* gene exhibits enhanced *P. infestans* susceptibility, suggesting a role for *Rcr3* in defense against *P. infestans*. Furthermore, in contrast to *C. fulvum, P. infestans* has evolved an Rcr3-inhibitory effector that avoids triggering a Cf-2-mediated HR (Song et al., 2009). In a yeast-two-hybrid screen and subsequent pull-down assays the venom allergen protein from the root nematode *Globodera rostochiensis* (Gr-vap1) was found to interact with Rcr3 along with various other host proteins that are associated with plant defense (Lozano et al., unpublished data). This demonstrates that on the one hand the same host protein can function as a target for multiple seemingly unrelated effectors, and also at the other hand one effector can target multiple host proteins.

2.2.4 RD19

The effector PopP2 (*Pseudomonas solanacearum* outer protein P2) of the soil-borne bacterial vascular pathogen *Ralstonia solanacearum* (previously known as *P. solanacearum)* interacts directly with its cognate RRS1-R (Resistance to *Ralstonia solanacearum1-R*) resistance protein of *A. thaliana* in the nucleus.

As discussed in chapter 1, the *A. thaliana* cysteine protease RD19 (Responsive to Dehydration 19) interacts with the effector PopP2 and it is suggested that this interaction forms a nuclear complex that is required for activation of the RRS1-R-mediated resistance response. Interestingly, both RD19 and PopP2 are cysteine proteases, with RD19 belonging to family C1 of PLCP Clan CA, and PopP2 to family C55 of YopJ-like Clan CE, respectively. However, it is unknown whether these cysteine proteases become activated after proteolytic processing by each other or one another (Bernoux et al., 2008).

Despite the absence of a nuclear localization signal (NLS) motif within the RD19 sequence, this protease is relocalized to the nucleus upon co-expression with

PopP2. Possibly Pop2 guides RD19 after binding to the nucleus by an unknown mechanism. However, nuclear localization of cysteine proteases without a NLS has been reported previously (Tabaeizadeh et al., 1995; Harrak et al., 2001; Goulet et al., 2004).

2.2.5 TDI-65

TDI-65 (tomato drought-induced 65) is a PLCP that carries a C-terminal granulin domain, that has been implicated as a regulator of cell growth (Bateman and Bennett, 1998; Yamada et al., 2001). *TDI-65* is induced by abiotic stress such as drought, heat, cold and during senescence (Schaffer and Fischer, 1990; Drake et al., 1996; Harrak et al., 2001). Immunolocalization studies showed that TDI-65 is involved in protein breakdown in chloroplasts and nuclei during drought stress (Tabaeizadeh et al. 1995). Interestingly, Avr2 interacts with TDI-65 probably with low affinity; this could indicate that TDI-65 is also involved in defense (van Esse et al., 2008).

3. Bacterial cysteine proteaes

Several effectors of bacterial plant pathogens have shown to be cysteine proteases that belong to various classes and act directly or indirectly against plant defense mechanisms.

AvrRpt2 is a cysteine protease which belongs to the C70 family and does eliminate Rin4, resulting in suppression of basal defense (Kim et al., 2005) as described in chapter 1.

A significant number of these effectors belong to the C55 family, such as the *R. solanacearum* effectors PopP1 and PopP2 and the *Xanthomonas* sp. effectors AvrXv4, AvrBsT and AvrRxv. All are characterized as YopJ-like SUMO (small ubiquitin-like modifier) proteases and are predicted to modify signal transduction pathways by removing SUMO modifications from elements involved in plant defense pathways (Orth et al., 2000).

The mammalian pathogen *Yersinia pseudotuberculosis* secretes the effector YopJ that interacts with MAPKs (mitogen-activated protein kinases) and plays a role in suppression of innate immunity, suggesting that like effectors of human pathogenic bacteria, also effectors of plant pathogenic bacteria might be involved in down-regulation of PTI signaling (Espinosa et al., 2004).

XopD was the first effector of this class which showed protease activity on SUMO-modified plant proteins, but not on SUMO-modified animal proteins (Hotson et al., 2003).

Another large family of TTSS effectors shared between plant and mammalian pathogens has famous members such as the *P. syringae* effectors HopAR1 (AvrPphB) and HopC1 and belong to the C58 family of proteases or YopT-like proteases (from *Yersinia pestis*) (Iriarte et al.,1998; Shao et al., 2002). HopAR1 cleaves PBS1 which is involved in negative regulation of the RPS5 resistance protein.

4. Protease inhibitors

Proteases are involved in numerous vital intracellular and extracellular processes and therefore it is essential to strictly control their activities in order to prevent unwanted harmful effects. In general, proteases are synthesized as large precursor proteins called zymogens or pre-pro-proteins. The pre-peptide functions as a signal peptide for localization, and activation occurs by removal of the inhibitory pro-segment. Thus the pro-peptide acts as an inhibitor to keep the enzyme inactive, pro-peptides are covering the active site of the protease (Bode et al., 2000). In this way, the cell prevents damage by uncontrolled protease activity.

Once activated, the enzymatic activity of a protease can be controlled and regulated by endogenous inhibitors that are found in the same cellular compartment (Clawson, 1996, Gomez et al., 1997). Protease inhibitors have been described for all classes of proteases, and are diverse types of proteinaceous compounds that can vary significantly in size (http://merops.sanger.ac.uk; Rawlings et al., 2010). Endogenous protease inhibitors contain usually domain(s) that enter or block the active site of the protease to prevent substrate access in a competitive manner. Also more complex modes of inhibition with intermediate steps are known. Non-competitive inhibitors bind to an allosteric site of the protease which modifies the global structure of the protease, and as a consequence the active site becomes inaccessible to the substrate. Uncompetitive inhibitors only interact with the protease when it is bound to the substrate thereby forming a trimeric complex (Bode et al., 2000).

Reversible and irreversible types of inhibition occur and endogenous inhibitors can be divided in three types: threshold type, buffer type and delay type. The threshold type inhibitors prevent accidental activation of a protease. Buffer type inhibitors always act in a reversible manner, and very swiftly associate and dissociate with the protease in absence or in presence of the substrate, respectively. Delay type inhibitors slowly bind to the active protease such that proteolysis only occurs for a limited period of time (Turk et al., 2005).

Remarkably, particular substrates such as serpins and baculovirus protein p35, only act as an inhibitor after cleavage during the interaction with the protease (Ye et al., 2001). Besides proteases that play a role in virulence, various pathogenic micro-organisms use protease inhibitors to inactivate proteases that play a role in host defense. In the following section I will focus on cysteine protease inhibitors in pathogen-plant interactions.

4.1 Cysteine protease inhibitors

Cystatins are a superfamily of cysteine protease inhibitors that can be divided in four families: stefins, cystatins, kinogens and phytocystatins. (Oliveira et al., 2003, Kordiš and Turk, 2009) Stefin cysteine protease inhibitors are small in size (about 100 amino acids), carry a single inhibiting domain and possess no disulphide bridges or glycosylation sites. Stefins usually reside in the cytoplasm, but some have reported

to occur extracellularly. Cystatins are proteins of about 120 amino acids in size, containing two disulphide bridges and a signal peptide for extracellular secretion. Today seven members have been described (Cystatin C, S, SA, SN, D, E/M and F). Cystatin F has an additional N-terminal disulphide bridge. A number of other members of the cystatin family do not show strong resemblance based on the inhibitory function (Abrahamson et al., 2003). Particularly the CRES (cystatin-related epididymial spermatogenic) proteins are found to inhibit serine proteases instead of cysteine proteases (Cornwall et al., 2003). The kininogen family is harboring members with multifunctional glycoproteins containing three tandemly repeated cystatin domains (Turk et al.,1996). Phytocystatins constitute a single branch that is statistically distinct from the animal cystatins and revealed the existence of a typical [Leu,Val,Ile]-[Ala,Gly,Thr]-[Arg,Lys,Glu]-[Phe,Tyr]-[Ala,Ser]-[Val,Ile]-Xxx-[Glu,Asp,Gln,Val]-[His,Tyr,Phe,Gly]-Asn consensus sequence also known as the LARFAV motif (Figure 4) (Margis et al., 1998; Margis-Pinheiro, et al., 2008).

4.1.1 Mechanisms of interaction

The resolution of the crystal structure of chicken cystatin was a major breakthrough in the elucidation of the mechanism of interaction between cystatins and PLCPs. Three regions of cystatin are involved in the interaction: the N-terminus and two hairpin loops that connect the β-sheet strands (Bode et al., 1988). Later on, this was confirmed by the resolution of the crystal structure of the stefin B-papain complex (Stubbs et al., 1990). Most cystatins are reversible competitive inhibitors. They are rather non-specific but bind firmly to their targets with Ki-values in the µM to pM range. The binding most likely occurs in a two step process; first the two loops binds to the protease and subsequently the N-terminus binds and enhances the contact. The cysteine present in the catalytic centre of the protease appears not to be involved in this interaction.

The typical Gln-Xxx-Val-Xxx-Gly motif and the C-terminal Trp residue are important in the affinity for the target protease. The N-terminal Gly residue is very important for the inhibition (Bode et al., 1988). Phytocystatins possess hypervariable sites located at strategic positions of the protein: on each side of the conserved Gly residue in the N-terminal part, within the Gln-Xxx-Val-Xxx-Gly motif and second Trp containing inhibitory loops entering the active site of target enzymes, and surrounding the LARFAV motif, a sequence of unknown function but conserved among plant cystatins (Urwin et al., 1995, Kiggundu et al., 2006).

4.2 Plant cysteine protease inhibitors

Plant cysteine protease inhibitors such as Oryzacystatin are involved in defense against insects and nematodes. A reduction of growth of insects has been observed when feeding on cystatin-overproducing plants, but also enhanced resistance to various potyviruses has been reported (reviewed by Dunaevsky et al., 2005; Haq et

al., 2004; Lawrence and Koundal, 2002; Gutierrez-Campos et al., 1999; Walker et al., 1999). So far no plant cysteine protease inhibitors involved in defense against microbial pathogens have been reported.

4.3 Pathogen cysteine protease inhibitors

Mining of the whole genome sequence of *P. infestans* revealed 18 extracellular protease inhibitor genes encoding two major structural classes: (i) Kazal-like serine protease inhibitors (Epi1 to Epi14) of which Epi1 and Epi10 inhibit the P69B subtilisin-like serine protease of tomato and (ii) cystatin-like cysteine protease inhibitors (EpiC1 to EpiC4) (Tian et al., 2004, 2005). EpiC1 and EpiC2B have been tested in detail and were found to interact with the extracellular tomato proteases Pip1 and Rcr3 (Tian et al., 2007).

In contrast to Avr2, the interaction of EpiC1 and EpiC2B with Rcr3 does not trigger a Cf-2-mediated HR. This can be explained based on sequence homology, since EpiC1 and EpiC2B contain all motifs typical for cystatins, whereas Avr2 does not. It is conceivable that EpiC1 and EpiC2B inhibit Rcr3 and Pip1 in a competitive manner, in contrast to the uncompetitive inhibition of Rcr3 by Avr2. It is very likely that the interaction of EpiC1 and EpiC2B with Rcr3 does not lead to Rcr3 modification, and thus does not trigger a Cf-2-mediated HR.

EpiC1 and EpiC2B are characterized as cystatins that show homology to plant cystatins, which makes it conceivable that these effectors mimic the endogenous plant cystatins in a way that remains undetected by the host surveillance system. The importance of EpiCs in *P. infestans* virulence remains yet to be documented.

The *C. fulvum* Avr2 effector protein appears to be a unique cysteine protease inhibitor which inhibits Rcr3 in a uncompetitive manner (van ‘t Klooster et al., 2010). This type of inhibition implicates that Avr2 is expected to bind to the Rcr3-substrate complex. To date the intrinsic Rcr3 substrate and substrate specificity is unknown.

PCD is a process involved in host defense (HR) and senescence, and Rcr3 is involved in both processes (Krüger et al., 2002), suggesting that the substrate and its products after processing by Rcr3 may be involved in those two phenomena also. Like CathB, Rcr3 might be involved in downstream signaling in defense and senescence.

5. Concluding remarks on the guard hypothesis and effector molecules

Indirect interactions between R proteins and effectors follow the guard model as postulated by Van der Biezen and Jones (1998), and subsequently extended by Dangl and Jones (2001), which implicates that an R protein acts as a guard that monitors the host target (the guardee) of the pathogen effector to monitor perturbations in its physical state. The guard hypothesis is well studied and characterized in pathosystems involving plant pathogenic bacteria. In order to colonize the apoplast of the host, bacterial pathogens deliver effector proteins into the plant cell by the TTSS where they interact with cytoplasmic virulence targets to interfere with basal host resistance and/or suppress PAMP-triggered immunity (PTI). Exactly these perturbations of the virulence targets are sensed by the guarding R proteins which activate effector-triggered immunity (ETI), a faster and stronger version of PTI that often culminates in the HR (Jones and Dangl, 2006). It is intriguing to observe the similarity and the geniality of "weapons" applied by both pathogen and host in attack and defense. Effectors of pathogens and host defense molecules are often enzymes which are dynamic molecules and can initiate a cascade of mechanisms. Activation or inhibition of enzymes can finally establish a successful infection or defense. Many enzymes are involved in processes like PCD. This requires a strict control of the enzymes in order to prevent harmful damage.

Cysteine protease and cysteine protease inhibitors play a distinct and crucial role in pathogen-plant interactions. It is also intriguing to indentify the substrates of specific cysteine proteases, which will provide more inside and detailed information about secondary or indirect mechanisms involved in pathogenicity and defense. Study of the tomato-*C. fulvum* pathosystem revealed that the Avr2-Rcr3-Cf2-mediated resistance complex complies with the guard hypothesis. The Rcr3 cysteine protease has been demonstrated to be the guardee in the resistasome complex, but is also an effector target of pathogens that are unrelated to *C. fulvum*, including an Oomycete and likely also a nematode (van Esse et al, 2008; Lozano et al., personal communication). This suggests that Rcr3 is a major player in host defense which is targeted by diverse types of pathogens to inhibit or to eliminate. So far, only tomato has developed an efficient guard (Cf-2) to eliminate the inhibitory activity of the Avr2 *fulvum* effector by mediating an HR; it also indicates that Avr2 is a unique cysteine protease inhibitor, for which tomato during evolution needed to get rid of. R gene-mediated resistance data are accumulating, which indicate that perception of pathogens and initiation of host defense is complex. In the R protein complex many accessory proteins take part forming a complex referred to as the resistasome. As such, the virulence target-R protein complex serves as a platform for many interacting proteins that are required for a tight control of the resistasome to prevent unnecessary HR.

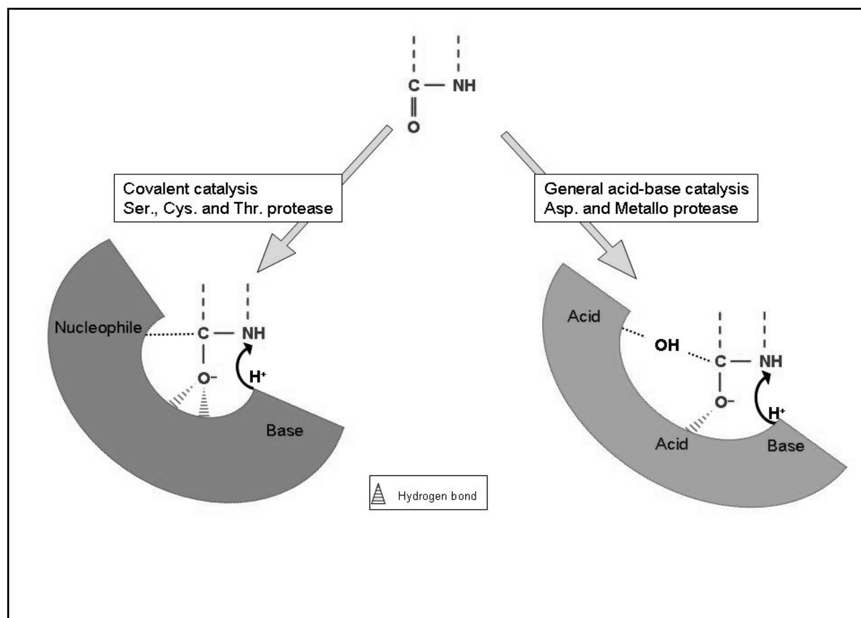

Figure 1. Catalytic mechanisms of different proteases.
(after Turk, 2006)
The five major catalytic classes of proteases can be divided in two groups based on fundamentally different catalytic mechanisms. In the covalent catalysis as occurs in the serine, cysteine and threonine proteases, the nucleophile of the catalytic site is represented by the side chain of an amino acid residue, whereas the His residue normally functions as a base.
In the non-covalent catalysis as in the metalloproteases and aspartic proteases, the nucleophile is an activated water molecule and the Asp or Glu residues and zinc (metalloproteases) serve as acids and base, respectively.
A further obvious difference between these two groups relates to the formation of the reaction products that for cysteine and serine proteases require an additional intermediate step (acyl-enzyme intermediate).

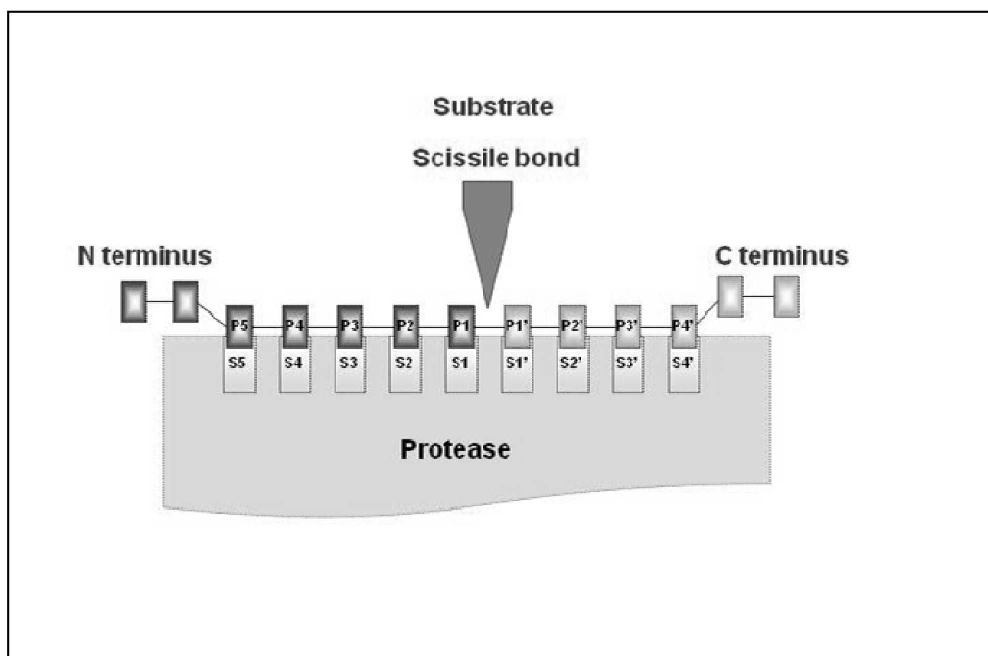

Figure 2. Schematic representation of a protein substrate binding to a protease.
(after Turk, 2006)
The subsite of a protease is the location of an amino acid residue that is able to accommodate a single side chain of a substrate residue. Beginning from the sites on each side of the scissile bond, the subsites are numbered S1–Sn upwards to the N terminus of the substrate (non-primed sites), and S1′–Sn′ towards the C terminus (primed sites). The facing amino acid residues of the substrate are numbered P1–Pn, and P1′–Pn′, respectively. The substrate specificity of a protease is determined by the structure of the active site of a protease thereby specifying which substrate residues can bind to specific substrate binding sites of the protease also known as the intrinsic subsite occupancy.

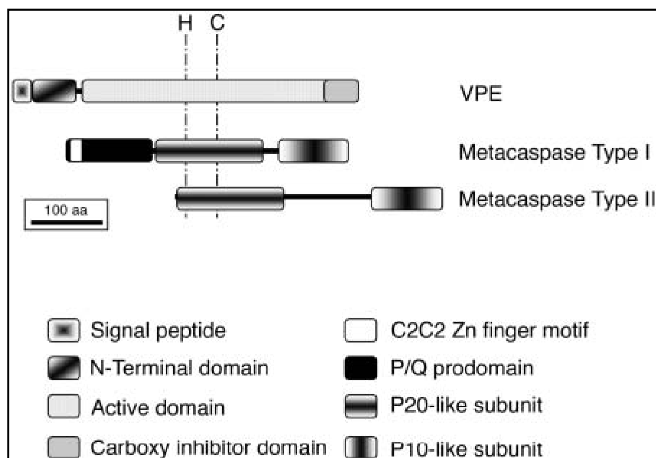

Figure 3. Plant VPEs and metacaspases.
(after Sanmatin et al., 2005)
The position of the His-Cys amino acid residue of catalytic dyad is shown.
Vacuolar processing enzymes (VPEs) are synthesized as inactive zymogens with a signal peptide for insertion into the endomembrane system. The C-terminal inhibitory domain and the N-terminal domain of VPEs are sequentially removed, most likely in the acidic environment of the vacuole.
Metacaspases are most likely cytosolic enzymes that may be autoprocessed into p20 and p10 subunits present in both types of metacaspases. Type I metacaspases contain an additional prodomain rich in Pro and Gln residues and a zinc finger motif.

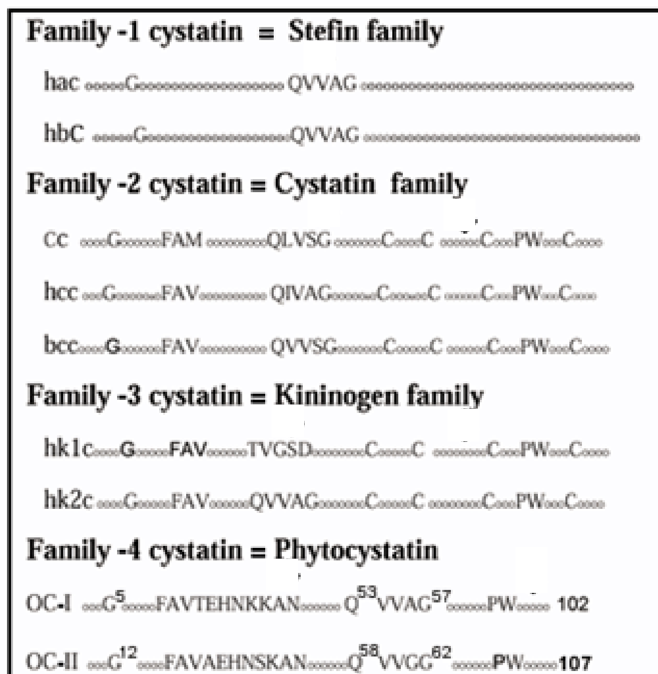

Figure 4. Alignment of amino acid residues specific for the four cystatin families.
(after Margis et al., 1998)
hac: human cystatin A
hbC: human cystatin B
Cc: chicken cystatin,
hcc: human cystatin C
bcc: bovine colostrum cystatin
hk1c: human kininogen segment 1
hk2c: human kininogen segment 2
OCI: Oryzacistatin-I
OCII: Oryzacistatin-II

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Summary

Plants are not able to move or escape and have to confront environmental challenges like nutrient and water deprivation, low and high temperatures, and biotic stress imposed by pathogens like viruses, bacteria, fungi, nematodes and insects that all compete for plant nutrient sources. The outcome of a plant-pathogen interaction can vary from mild symptoms that are hardly harmful to the host to complete destruction of the host plant. Plants have evolved various mechanisms to counter-attack infections by pathogens. Mechanisms of evasion or suppression of basal host defense by pathogens on the one hand, and specific recognition of a pathogen by its host and activation of downstream defense signaling on the other hand, are complex and both organisms have to come up with sophisticated strategies to survive their encounters. In principle these encounters have two possible outcomes: (i) a pathogen successfully infects the host plant, which is also referred to as a compatible interaction (the pathogen is virulent and the host plant is susceptible), or (ii) the pathogen cannot successfully infect the host plant which stays healthy, also referred to as an incompatible interaction (the pathogen is avirulent and the host plant is resistant).

Nearly 70 years ago, Harold Flor (1942) studied the genetics of the interaction between the flax rust fungus *Melampsora lini* and flax, *Linum usitatissimum*. Based on these studies he postulated the so-called gene-for-gene hypothesis (Flor 1942) which states that for each dominant resistance (*R*) gene in the host there is a matching dominant avirulence (*Avr*) gene in the pathogen. Co-occurrence and expression of both genes leads to an incompatible interaction that is often associated with a hypersensitive response (HR).

The interaction between the fungus *Cladosporium fulvum* (syn. *Passalora fulva*) and the host tomato *(Solanum lycopersicum)* is an excellent model to study plant-pathogen interactions and obeys to the gene-for-gene hypothesis. *C. fulvum* is a biotrophic pathogen that causes leaf mold of tomato, avoids breaching the cell wall and exclusively colonizes the tomato leaf apoplast while establishing a long-term feeding relationship with the living cells of the host. During the infection process, the fungus secretes several effector molecules, relatively small, cysteine–rich proteins. They are likely to contribute to pathogen fitness and play a role in pathogen virulence. According to the 'Zig-Zag' model that explains the evolutionary development of plant-pathogen interactions, effectors are required for ETS (effector - triggered susceptibility). Tomato plants that carry cognate *Cf* resistance genes recognize the effector and elicit a defense response known as the hypersensitive response (HR), nowadays known as effector-triggered immunity (ETI). In this thesis I have focused on several molecular and biochemical aspects of the *Avr2* and *Cf-2* gene pair and on an additional gene, *Rcr3* (required for Cladosporium resistance),

that is required for Cf-2-mediated resistance with an emphasis on the role of Avr2 in ETS and ETI in the *C. fulvum*-tomato interaction.

The *gene*-for-*gene* hypothesis postulated by Harrold Flor has inspired many plant pathologists and initiated numerous plant-pathogen studies as discussed in **chapter 1**. This hypothesis has lead to the characterization of various host plant *R* genes and cognate pathogen *Avr* genes from fungi, bacteria and oomycetes. Plant resistance proteins are the basic molecules that mediate a defense reaction, triggered by cognate effectors directed against the pathogens, are found extracellularly as well as intracellularly and are divided in classes based on the composition of different subdomains that may have various functions. Particularly the LRR domain(s) are involved in recognition, regulating protein activation and signal transduction and are highly adjustable in diverse binding specificities to self and non-self molecules. The nucleotide binding (NB) domain acts a switch for activation of downstream host defenses, often resulting in HR. Inappropriate R protein folding and activation is controlled by intramolecular interactions between the various domains and by hetero-multimeric protein complexes.
Studies on interactions of plants, especially *Arabidopsis thaliana*, with prokaryotic pathogens have resulted in major scientific breakthroughs with respect to the gene-for-gene hypothesis. Research on the bacterial Type Three Secretion System and the delivery of the effectors has indentified sophisticated mechanisms for perception and recognition of pathogens and regulation of host resistance.
The functions of effectors of eukaryotic plant pathogens remain largely unknown so far. Oomycete pathogens such as *Phytophthora infestans* produce various types of effectors during infection of their hosts. One class of oomycete effectors localizes to, and operates in, the extracellular matrix while the other class acts inside the host plant cell. Recent studies on the interaction of the flax rust fungus *Melampsora lini* with flax (*Linum usitatissimum*) has revealed a number of direct Avr-R protein interactions *in vitro.* These interactions are expected to occur in the haustorial matrix which is produced by the fungus during host infection. Secreted Avr proteins of *C. fulvum* interact exclusively with the corresponding extracellular Cf proteins of tomato. The *C. fulvum*-tomato pathosystem is one of the most well-studied plant pathogen interactions and revealed important insights in perception and recognition of Avr proteins. For many years it was assumed that the interactions beween *C. fulvum* Avrs and tomato R proteins occurred in a direct manner, but proof for such interactions has never been obtained. Indirect interactions were more likely and obeyed to the guard hypothesis wherein the Avr protein interacts with a host target and this interaction is monitored, or guarded, by the Cf- protein.

Chapter 2 reports on the avirulence function of Avr2 in the Cf-2-mediated resistance that also requires the extracellular tomato cysteine protease Rcr3. The interaction between Avr2, Cf-2 and Rcr3 obeys to the guard hypothesis. Purified heterologously expressed and affinity-tagged Rcr3 and Avr2 were applied in co-immunoprecipitation assays and revealed a physical interaction between Avr2 and Rcr3 independent of additional plant and or fungal factors. It is shown that Avr2 binds and inhibits Rcr3, and blocking of the active site of Rcr3 by the irreversible cysteine protease inhibitor E-64 eliminates this interaction. The interaction with and the inhibition of Rcr3 by Avr2 occurs in a pH-dependent fashion and the pH optimum for Rcr3 activity and its inhibition by Avr2 coincides with the pH of the tomato apoplast. Cysteine protease activity profiling showed that, in addition to Rcr3, Avr2 inhibits several other apoplastic cysteine proteases in tomato, but this inhibition did not lead to Cf-2-mediated HR. Infiltration of purified active Rcr3, or E-64-inactivated Rcr3, in combination with Avr2 in *Cf-2*/*rcr3* tomato leaves revealed that only the Avr2-Rcr3 inhibition complex triggers Cf-2-dependent HR. It is proposed that Avr2 modifies Rcr3 which is recognized by Cf-2 and initiates the HR. This study represents the first indirect fungus-plant gene-for-gene interaction that obeys to the guard hypothesis.

In **chapter 3** the focus is on the virulence function of Avr2, and it is demonstrated that Avr2 has an indisputable intrinsic biological virulence function for *C. fulvum* during infection of tomato. Silencing of the *Avr2* gene in *C. fulvum* significantly compromised fungal virulence on tomato. Heterologous expression of *Avr2* in tomato resulted in enhanced susceptibility towards natural Avr2-defective *C.fulvum* strains, but also towards *Botrytis cinerea* and *Verticillium dahliae*. In *A. thaliana*, *Avr2* expression resulted in enhanced susceptibility to various extracellular fungal pathogens including *Botrytis cinerea* and *Verticillium dahliae*. Microarray analysis of unchallenged *A. thaliana* plants showed that *Avr2* expression induced a global transcription profile that is comparable to the profile upon pathogen challenge. Cysteine protease activity profiling and LC-MS/MS analyses showed that Avr2 inhibits multiple extracellular *A. thaliana* cysteine proteases. Similar results were obtained for tomato, showing that Avr2 inhibits multiple cysteine proteases including Rcr3 and its close relative Pip1. This all shows that Avr2 is a genuine virulence factor of *C. fulvum* that inhibits several cysteine proteases that are required for basal host defense.

In **chapter 4** the emphasis is on Avr2 protein features and the mode of inhibition of Rcr3. Like many other *Avr* genes, *Avr2* lacks homology with sequences deposited in public databases. The mature Avr2 protein contains 8 cysteine residues and biochemical analyses revealed that all of these are involved in disulphide bridging, showing a unique disulphide bridge pattern. Based on a bioinformatics analysis, site-specific mutations were made in the Avr2 protein and affinity-tagged wild-type and mutant proteins were produced by heterologous expression in the yeast *Pichia pastoris*. After affinity purification, all proteins were infiltrated in tomato *Cf-2* plants, and proteins with altered HR inducing activity were tested for their ability to inhibit Rcr3. From these assays it became evident that especially the C-terminal six amino acids that also include one disulphide bridge are essential for the interaction with and inhibition of Rcr3. All these data show that Avr2 is a novel type of cysteine protease inhibitor.

Chapter 5 is a general discussion about the role of plant cysteine proteases and cysteine protease inhibitors in plant-pathogen interactions. Microbial pathogens and host plants both employ cysteine proteases and cysteine protease inhibitors as weapons for attack and defence. This so-called arms race has led to multiple attacks and counter-attacks that have shaped co-evolution between pathogens and their host plants. Examples of some prokaryotic plant pathogens that employ cysteine proteases as effector proteins to suppress plant defense will be discussed, in addition to some eukaryotic pathogens that use cysteine protease inhibitors for the same purpose. Examples of plant cysteine proteases will be discussed that are involved in multiple processes including plant development, plant defense and processes in programmed cell death.

Samenvatting

Planten zijn plaatsgebonden en kunnen niet vluchten wanneer er gevaar dreigt. Ze worden niet alleen voortdurend blootgesteld aan abiotische stressfactoren, zoals te kort aan voedingsstoffen en water, UV-straling en grote temperatuurverschillen, maar ook aan biotische stressfactoren zoals pathogene virussen, bacteriën, schimmels, aaltjes en insecten die allemaal de plant als bron van voedingsstoffen gebruiken. De uitkomst van een plant-pathogeen interactie kan variëren van milde symptomen tot de dood van de aangetaste plant. Tijdens de evolutie hebben planten diverse mechanismen ontwikkeld om zich te verweren tegen pathogenen, en pathogenen hebben op hun beurt mechanismen ontwikkeld om de afweer van planten te omzeilen of te onderdrukken. Deze wisselwerking is complex en beide organismen moeten voortdurend nieuwe strategieën ontwikkelen om te overleven. In principe kunnen genoemde confrontaties eindigen in (i) een succesvolle infectie van de plant door het pathogeen, of (ii) een geslaagde afweer van de plant tegen het pathogeen. Wanneer de plant overwint wordt hij resistent genoemd, het pathogeen avirulent en de interactie incompatibel. Wanneer het pathogeen overwint wordt het virulent genoemd, de plant vatbaar, en de interactie compatibel.

Bijna 70 jaar geleden heeft Harrold Flor op basis van genetische studies aan de interactie tussen de vlasroest schimmel *(Melampsora lini)* en zijn waardplant vlas (*Linum usitatissimum),* de gen-om-gen hypothese opgesteld. Deze hypothese stelt dat bij een incompatibele interactie voor elk dominant resistentiegen (*R*) in de waardplant een dominant avirulentiegen (*Avr*) in het pathogeen aanwezig is. Expressie van *R* gen en bijpassend *Avr* gen leidt tot interactie tussen hun producten, hetgeen meestal gepaard gaat met een overgevoeligheidsreactie of hypersensitieve respons (HR) die de groei van het pathogeen blokkeert in de waardplant.

De interactie tussen de schimmel *Cladosporium fulvum (*syn*. Passalora fulva*) en zijn waardplant tomaat *(Solanum lycopersicum),* is een modelsysteem geworden voor bestudering van plant-pathogeen interacties waarvoor de gen-om-gen hypothese geldt. *C. fulvum* veroorzaakt de bladvlekkenziekte van tomaat. De schimmel koloniseert alleen de apoplast van tomatenbladeren en produceert geen voedingslichaampjes (haustoria) in de mesofylcellen van tomaat. Op deze manier wordt een intieme relatie aangegaan met de mesofylcellen waarbij de schimmel zich vrijwel onopgemerkt lijkt te kunnen voeden op levende cellen (biotrofe levenswijze), waarschijnlijk omdat hij het basale immuunsysteem van tomaat kan onderdrukken door de productie van effectoren die hij tijdens infectie van de plant uitscheidt. Effectoren zijn relatief kleine, cysteine-rijke, eiwitten die, naast het onderdrukken van het basale immuunsysteem van de plant, ook van belang zijn voor de algemene fitheid van de schimmel.

Volgens het 'Zig-Zag' model, dat de evolutionaire ontwikkeling van plant-pathogeen interacties beschrijft, onderdrukken effectoren de zogenaamde pathogeen-geassocieerde moleculaire patroon (PAMP)-geïnduceerde immuniteit (PTI) hetgeen leidt tot effector-geïnduceerde vatbaarheid (ETS) en aantasting van de waardplant. Resistente tomatenplanten bezitten *Cf* resistentiegenen die effectoren kunnen herkennen en reageren met een HR, ook wel aangeduid als effector-geïnduceerde immuniteit (ETI).

In dit proefschrift is het onderzoek vooral gericht op moleculair-genetische en biochemische aspecten van het *Avr2-Cf-2* genenpaar, en een additioneel gen, *Rcr3* (required for Cladosporium resistance), dat codeert voor een cysteine protease dat nodig is voor Avr2-geïnduceerde Cf-2-gemedieerde resistentie. De nadruk ligt op de rol van Avr2 in ETS en ETI.

De gen-om-gen hypothese, zoals opgesteld door Harrold Flor, heeft door de jaren heen vele moleculaire fytopathologen geïnspireerd om onderliggende mechanismen op te helderen met als gevolg en dat een groot aantal plant-pathogeen modelsystemen bestudeerd zijn waarvan een overzicht gegeven is in **hoofdstuk 1**. De studies hebben er toe geleid dat er gedurende het afgelopen decennium vele *R* genen en bijpassende *Avr* genen van bacteriën, schimmels en oomyceten zijn geïdentificeerd en gekarakteriseerd. R eiwitten spelen een centrale rol bij afweer, omdat ze effectoren van pathogenen herkennen waarna afweerreacties aangeschakeld worden. Deze afweerreacties zijn gericht tegen het pathogeen en vinden zowel extracellulair als intracellulair plaats. R eiwitten kunnen onderverdeeld worden in klassen gebaseerd op aanwezigheid van verschillende subdomeinen die verschillende functies bezitten. Vooral de leucine-rijke 'repeats' (LRRs) komen vaak voor in R eiwitten en zijn vooral betrokken bij eiwit-eiwit interacties waaronder de herkenning van zowel eigen als vreemde eiwitten. Zo spelen ze ook een rol bij de herkenning van effectoren en de daaropvolgende activering van signaal transductie ketens betrokken bij de afweer van de plant.

Veel R eiwitten bezitten ook een nucleotide binding (NB) domein dat werkt als een schakelaar bij de activering van signaal transductie ketens die vaak uitmonden in een HR. Intramoleculaire interacties tussen de verschillende domeinen en heteromultimere eiwitcomplexen spelen een rol bij de controle op onjuiste vouwing en ongewenste activering van R eiwitten.

Studies aan interacties tussen vooral zandraket (*Arabidopsis thaliana)* en prokaryote pathogenen hebben de afgelopen jaren tot belangrijke wetenschappelijke doorbraken geleid in ons begrip van gen-om-gen interacties. Onderzoek naar secretiemechanismen van bacteriële effectoren heeft geleid tot de identificatie van complexe mechanismen die van belang zijn bij herkenning van bacteriën door planten en bij de regulatie van waardplantresistentie.

Tot op heden zijn de functies van de meeste effectoren van de eukaryote plantenpathogenen nog onbekend. Pathogenen behorende tot de oömyceten, zoals

de verwekker van de aardappelziekte, *Phytophthora infestans,* produceren honderden effectoren tijdens infectie van hun waarplant. Eén klasse van effectoren van oömyceten bezit het zogenaamde RXLR domein dat een belangrijke rol speelt bij de opname in waardplantcellen volgens een nog onbekend mechanisme.
Recentelijk onderzoek aan Avr en R eiwitten van respectievelijk de vlasroestschimmel (*Melampsora lini)* en vlas (*Linum usitatissimum*) heeft aangetoond dat deze eiwitten fysiek interacteren *in vitro.*

Avr eiwitten van *C. fulvum* worden uitgescheiden in de apoplast van tomaat en interacteren met hun corresponderende extracellulaire Cf eiwitten in tomaat. Het *C. fulvum*-tomaat pathosysteem is een van de best bestudeerde plant-pathogeen interacties en heeft geleid tot belangrijke inzichten in de functie en herkenning van Avr eiwitten. Lange tijd werd aangenomen dat de Avr eiwitten van *C. fulvum* direct interacteren met de Cf eiwitten van tomaat, maar experimenteel bewijs hiervoor is tot op heden nog niet gevonden. Indirecte interacties zijn meer waarschijnlijk en passen ook in de zogenaamde bewakertheorie, ook wel "guard hypothesis" genoemd, waarbij het Avr eiwit een interactie aangaat met een virulentie-eiwit in de waardplant dat bewaakt wordt door het Cf- eiwit.

Hoofdstuk 2 beschrijft de avirulentiefunctie van Avr2 in de Cf-2-gemedieerde resistentie waarvoor ook het extracellulaire Rcr3 cysteine protease van de tomaat vereist is. De interactie tussen Avr2, Cf-2 en Rcr3 past in de "guard hypothesis". Rcr3 en Avr2 eiwitten, voorzien van affiniteitlabels, werden heteroloog tot expressie gebracht, gezuiverd en gebruikt in co-immunoprecipitatie assays. Deze toonden aan dat Avr2 en Rcr3 een fysieke interactie aangaan onafhankelijk van andere planten- en/of schimmelfactoren. Binding van Avr2 aan het Rcr3 cysteine protease remt zijn enzymatische activiteit. Het blokkeren van het catalytisch centrum van Rcr3 met de irreversibele cysteine protease remmer, E-64, wordt geremd door Avr2, hetgeen een directe aanwijzing is voor interactie met, en remming van, Rcr3 door Avr2. De interactie met, en de remming van, Rcr3 door Avr2 is pH-afhankelijk; het pH optimum voor de Rcr3 activiteit en de remming door Avr2 komt overeen met de pH van de apoplast van tomatenblad. Profilering van de cysteine protease activiteit toonde aan dat, naast Rcr3, Avr2 ook een aantal andere apoplastische cysteine proteases van tomaat remt, maar remming van deze proteases leidt niet tot een Cf-2-gemedieerde HR. Infiltratie van gezuiverd actief Rcr3, of E-64-geïnactiveerd Rcr3, in combinatie met Avr2 in *Cf-2/rcr3* tomatenbladeren liet zien dat alleen het Avr2-Rcr3 remmingscomplex Cf-2-gemedieerde HR kan induceren. Het is mogelijk dat Avr2 als gevolg van zijn interactie met Rcr3 het enzym modificeert, hetgeen wordt herkend door Cf-2 dat vervolgens de HR activeert. Deze studie geeft voor het eerst experimenteel bewijs voor een indirecte interactie tussen een schimmel Avr eiwit en bijbehorend plant R eiwit zoals voorgesteld in de "guard hypothesis".

In **hoofdstuk 3** wordt de virulentiefunctie van de Avr2 effector voor *C. fulvum* beschreven tijdens infectie van tomaat. Door het onderdrukken van de *Avr2* genexpressie in *C. fulvum* wordt de schimmel beduidend minder virulent op tomaat. Heteroloog tot expressie brengen van *Avr2* in tomaat resulteert in een verhoogde vatbaarheid niet alleen voor natuurlijke Avr2-deficiënte *C.fulvum* isolaten, maar ook voor twee andere pathogenen van tomaat, *Botrytis cinerea* en *Verticillium dahliae.* Expressie van *Avr2* in *A. thaliana* resulteert ook in verhoogde vatbaarheid voor *B. cinerea*.
Microarray analyses van niet geïnfecteerde Avr2 tot expressie brengende *A. thaliana* planten laten een globaal genexpressie profiel zien dat vergelijkbaar is met dat van geïnfecteerde planten.
Cysteine protease activiteitsprofilering en LC-MS/MS analyses toonden aan dat Avr2 meerdere extracellulaire cysteine proteases van *A. thaliana* remt. Vergelijkbare resultaten werden met tomaat verkregen waarbij Avr2, naast Rcr3, ook het nauw verwante Pip1 remt. Al deze gegevens bevestigen dat Avr2 een virulentiefactor voor *C. fulvum* is die verscheidene cysteine proteases remt die nodig zijn voor basale afweer.

In **hoofdstuk 4** zijn de eigenschappen van het Avr2 eiwit nader onderzocht met betrekking tot zijn vermogen om Rcr3 te remmen en Rcr3-afhankelijke Cf-2-gemedieerde HR te induceren. Zoals vele andere *Avr* genen van *C. fulvum*, vertoont *Avr2* geen homologie met bekende sequenties in publieke databanken. Het rijpe Avr2 eiwit bevat acht cysteine residuen, en met biochemische analyses werd bepaald dat deze alle acht een zwavelbrug vormen volgens een uniek patroon. Op basis van bioinformatische analyses, werden specifieke mutaties gegenereerd in het Avr2 eiwit en met behulp van het *Pichia pastoris* heterologe expressiesysteem werden wildtype Avr2 eiwit en een aantal mutante Avr2 eiwitten geproduceerd die voorzien waren van affiniteitlabels.
Na affiniteitszuivering werden alle eiwitten geïnfiltreerd in Cf-2 tomatenplanten en de eiwitten die een veranderde HR-inducerende activiteit vertoonden werden vervolgens getest op hun vermogen om Rcr3 protease-activiteit te remmen. Uit de resultaten kwam naar voren dat vooral de zes C-terminale aminozuren van het Avr2 eiwit zeer belangrijk zijn voor zowel de HR-inducerende activiteit als voor de remming van Rcr3. Al deze resultaten wijzen er op dat Avr2 een nieuw type cysteine protease remmer is.

In **hoofdstuk 5** worden alle resultaten globaal bediscussieerd, en wordt in het bijzonder de rol van cysteine proteases en cysteine protease remmers bij plant-pathogeen interacties belicht. Microbiële pathogenen en waardplanten gebruiken zowel cysteine proteases als cysteine protease remmers als wapens bij aanval en verdediging. Deze wapenwedloop heeft geleid tot het ontstaan van meerdere aanvals- en verdedigingsstrategiën tijdens de co-evolutie tussen pathogenen en hun waardplanten. Er worden voorbeelden van enige prokaryotische plantenpathogenen besproken die cysteine protease effectoren produceren om de plantenafweer te onderdrukken. Eveneens worden voorbeelden van enkele eukaryotische pathogenen besproken die juist cysteine protease remmers produceren om hetzelfde effect te bewerkstelligen. Ook enkele voorbeelden van plant cysteine proteases die een rol spelen bij plantontwikkeling en geprogrammeerde celdood worden bediscussieerd.

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Deze koele drieëntwintigjarige politieagent komt zomaar spontaan door die deur binnenvallen en dan word ik weer eens wakker en blijkt dat er ook nog een heel andere wereld daar buiten die deur is. Een wereld die ver van wetenschap staat maar waar wel elke dag over gepubliceerd wordt. Verfrissend waren de gesprekken hierover. Binnenkort toch maar eens samen met de motor op pad. Kerel, bedankt.

Henk Smid - Unifarm
Prachtig zijn ze, die foto's met de symptomen die wij Fytonezen zo graag willen zien. De testplant als het bewijs voor theorie. En dat kan alleen als de planten in sublieme conditie zijn en daarvoor zorgen de mensen van Unifarm. Voor mijn plantproeven heeft Henk Smid dat gedaan. Eén e-mailtje en een extra lamp was al aangesloten, meer of minder tomatenplanten, de "wilde" tomatenplanten, noem het maar op. Henk heeft dat voor mij geregeld en verzorgd. Een luxe was het om zo op je te kunnen vertrouwen. Mijn oprechte dank hiervoor.

Sjef en Jacques, De bioCHEMICI
Jullie kijken naar de eiwitten met een andere bril: vragen zoals welke zouten zitten in de oplosbuffer? Hoe zuiver is het preparaat?
Sjef Boeren, altijd gepassioneerd bezig om de LC-MS/MS te optimaliseren en daar ben ik je zeer dankbaar voor, maar ook: *Koffietijd* en *Lunchtijd*, om mij los te koppelen van de PC met al die ladingen aan data. Jacques Vervoort keek naar de eiwit-eiwit-interacties die voor mij ook weer nieuw waren. Veel heb ik van jullie geleerd en daarvoor mijn dank.

Jules Beekwilder
De wereld van de enzymen: Jules heeft mij wegwijs gemaakt in deze wereld van enzymen EN de remmers hiervan. Hierdoor ben ik gepassioneerd geraakt voor de dynamiek van deze interacties. Dat allemaal heb ik aan jou te danken Jules, heel veel dank hiervoor.

Studenten
Dan hebben we de WUR-studenten: Marc van der Kamp, die de basis heeft gelegd voor hoofdstuk 4. Verdere uitvoering hiervan is verricht door Eelco Hoogwout. *Jullada Laothawornkitkul has performed a major piece of work by generating a vast number of Avr2-mutant constructs*.
Daarna kwam er een reeks aan HAN/HBO-studenten: Mark de Graaf, Noor van Reekum, Nora Ludwig en Richelle Spanjers hebben veel testen verricht voor het vooronderzoek, maar zoals zo vaak loopt het onderzoek toch iets anders dan de hypothese beschrijft. Toch, zonder jullie inzet hadden we dat ook weer niet geweten. Mijn dank hiervoor is groot.
EN...via deze HAN-studenten ben ik uiteindelijk aan mijn huidige functie gekomen.

De HAN-collega's
Kort en krachtig kan ik zeggen: mijn huidige baan voelt als een warm nest. Toen eenmaal de definitieve beslissing was genomen om mijn promotie daadwerkelijk af te ronden, was ik verrast door de vele spontane en hartverwarmende reacties. Ook heb ik heerlijk kunnen ventileren als dat weer eens nodig was. Beste collega's, hartelijk dank hiervoor.

Pa en Ma Janssen
Ondanks dat mijn onderzoek soms te ver van jullie realiteit stond, hebben jullie zeker warme belangstelling hiervoor getoond. Een mengeling van trots en bezorgdheid. Regelmatig werd ik gevraagd voor een klusje bij jullie, zodat ik mijzelf losmaakte van de computer. En dan even bijkletsen en na een lekker *bakkie* koffie kon ik er weer tegenaan.
Pap en Mam, BEDANKT.

Ons El, thuis

Na een week Fyto, vrijdagsavonds, donker is het op 's lands wegen.
De motor spint, het dashboard met alle kleuren, geeft deze rit zijn zegen.
De economische levensader A50, A73 niet naar Köln. Nijmegen.
Als een landingsbaan, heldere straatverlichting in de verte, de skyline als decor.
Druk eraf. Het toerental zakt. Banden glijden in het asfaltspoor.
De bebouwde kom, garage inrollen, laatste meters voordat ik je stem weer hoor.
Keuken een bende, de geur van spaghetti. Dan weet ik dat we thuis zijn.
Een glas wijn, mijn bordje weer gevuld. Dan weet je dat we thuis zijn.
Uitbuiken op de bank. Voor het scherm met DVD. Dan voelen we dat we thuis zijn.
Fles is leeg. Loom wordt het lijf. Voor morgen is de rest.
Laatste ronde. Het huis op slot. Als twee honden in een nest.
Duizenden dankwoorden, in één blik naar jou. Dat weet jij als de beste.

Curriculum vitae

Johannes Wilhelmus (John) van ’t Klooster werd geboren op 27 november 1958 te Afferden, Gelderland. Na de middelbare school te Druten afgerond te hebben in 1975, begon hij in datzelfde jaar aan de opleiding HBO-analytische chemie te Oss die hij in 1978 succesvol heeft afgerond. Hierna heeft hij zijn militaire dienstplicht vervuld. In 1983 trad hij als analist in dienst bij de afdeling Bloedtransfusiedienst van het St.Radboud ziekenhuis te Nijmegen. Hier heeft hij zich verder ontwikkeld tot onderzoeksanalist bij de afdeling Transplantatieserologie, en in die tijd heeft hij de certificaten HLO- immunologie en moleculaire genetica behaald. In november 1989 trad hij als onderzoeksanalist in dienst bij de toenmalige vakgroep Veefokkerij van de Landbouwuniversiteit, Wageningen. In deze functie heeft hij zich verder gespecialiseerd in immunologische, biochemische en moleculair biologische technieken. Op 1 januari 1991 trad John in dienst als onderzoeksassistent bij de vakgroep Fytopathologie van dezelfde universiteit en heeft daar deel uitgemaakt van drie onderzoeksgroepen die werkzaam waren aan de plantenpathogenen *Phytothpthora infestans, Botrytis cinerea* en respectievelijk *Cladosporium fulvum.* Hier heeft hij hoofdzakelijk fundamenteel wetenschappelijk onderzoek verricht aan plant-pathogeen interacties met een moleculair biologisch en biochemisch karakter. Verder is hij betrokken geweest bij diverse onderwijsactiviteiten van de vakgroep Fytopathologie. Op 1 januari 2005 is hij begonnen aan zijn promotie-onderzoek over de rol van Avr2 en Rcr3 in Cf-2-gemedieerde resistentie van tomaat tegen *Cladosporium fulvum* in het kader van een EU project (BioExploit). De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Sinds september 2008 is John werkzaam als docent bij de HAN University of Applied Sciences te Nijmegen.

Publicaties als eerste auteur

John van 't Klooster, Vivianne G.A.A. Vleeshouwers, Sophien Kamoun and Francine Govers. (1999) Characterization of a cDNA Encoding a Pathogenesis Related Protein PR1 from Potato (*Solanum tuberosum*) (Accession No. AJ250136). Plant Gene Register PGR99-182 Plant Physiology, 121

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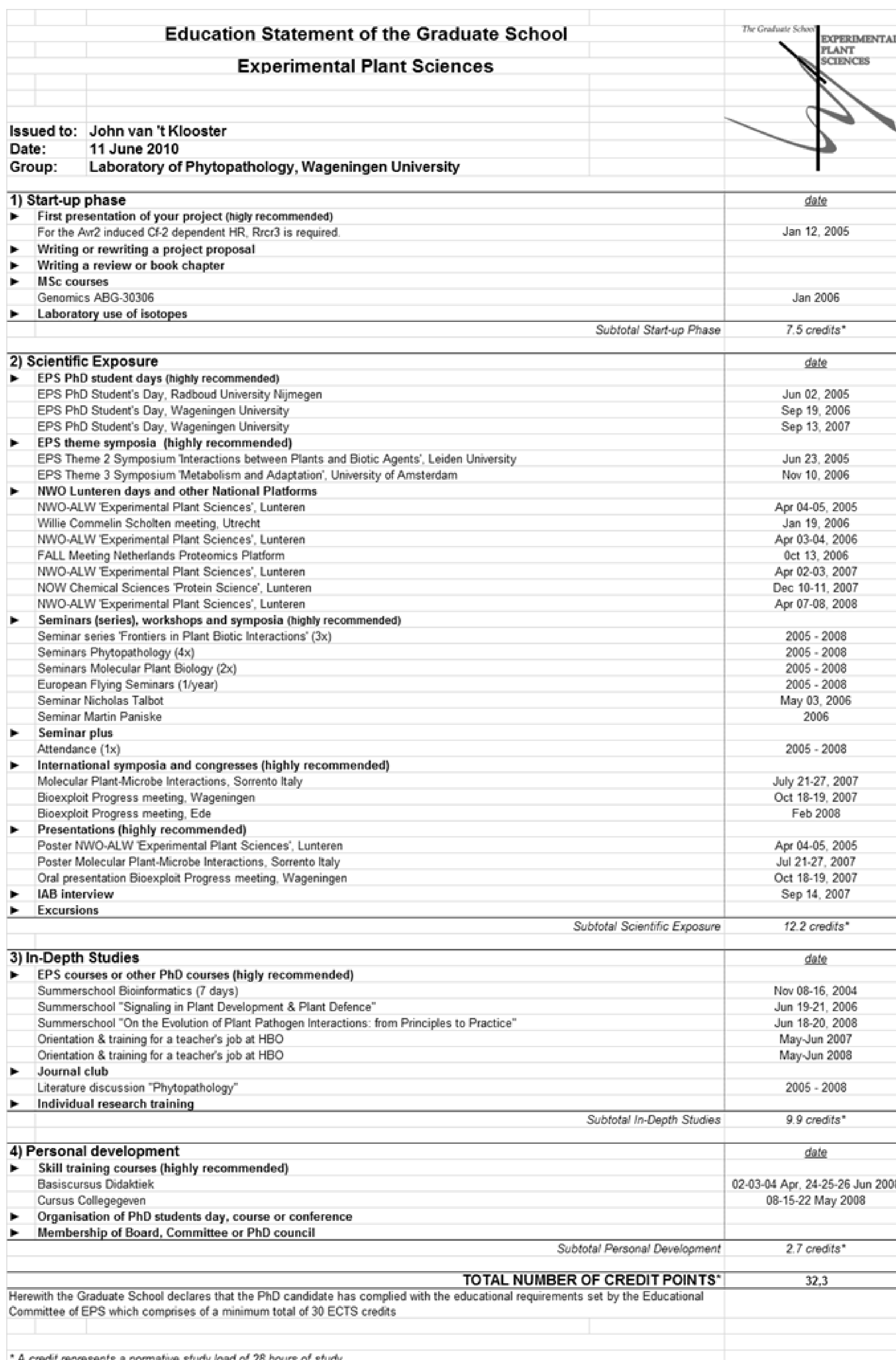

The Graduate School EXPERIMENTAL PLANT SCIENCES

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: John van 't Klooster
Date: 11 June 2010
Group: Laboratory of Phytopathology, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project (higly recommended)**	
For the Avr2 induced Cf-2 dependent HR, Rrcr3 is required.	Jan 12, 2005
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Genomics ABG-30306	Jan 2006
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days (highly recommended)**	
EPS PhD Student's Day, Radboud University Nijmegen	Jun 02, 2005
EPS PhD Student's Day, Wageningen University	Sep 19, 2006
EPS PhD Student's Day, Wageningen University	Sep 13, 2007
► **EPS theme symposia (highly recommended)**	
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Leiden University	Jun 23, 2005
EPS Theme 3 Symposium 'Metabolism and Adaptation', University of Amsterdam	Nov 10, 2006
► **NWO Lunteren days and other National Platforms**	
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2005
Willie Commelin Scholten meeting, Utrecht	Jan 19, 2006
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 03-04, 2006
FALL Meeting Netherlands Proteomics Platform	0ct 13, 2006
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2007
NOW Chemical Sciences 'Protein Science', Lunteren	Dec 10-11, 2007
NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
► **Seminars (series), workshops and symposia (highly recommended)**	
Seminar series 'Frontiers in Plant Biotic Interactions' (3x)	2005 - 2008
Seminars Phytopathology (4x)	2005 - 2008
Seminars Molecular Plant Biology (2x)	2005 - 2008
European Flying Seminars (1/year)	2005 - 2008
Seminar Nicholas Talbot	May 03, 2006
Seminar Martin Paniske	2006
► **Seminar plus**	
Attendance (1x)	2005 - 2008
► **International symposia and congresses (highly recommended)**	
Molecular Plant-Microbe Interactions, Sorrento Italy	July 21-27, 2007
Bioexploit Progress meeting, Wageningen	Oct 18-19, 2007
Bioexploit Progress meeting, Ede	Feb 2008
► **Presentations (highly recommended)**	
Poster NWO-ALW 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2005
Poster Molecular Plant-Microbe Interactions, Sorrento Italy	Jul 21-27, 2007
Oral presentation Bioexploit Progress meeting, Wageningen	Oct 18-19, 2007
► **IAB interview**	Sep 14, 2007
► **Excursions**	
Subtotal Scientific Exposure	*12.2 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses (higly recommended)**	
Summerschool Bioinformatics (7 days)	Nov 08-16, 2004
Summerschool "Signaling in Plant Development & Plant Defence"	Jun 19-21, 2006
Summerschool "On the Evolution of Plant Pathogen Interactions: from Principles to Practice"	Jun 18-20, 2008
Orientation & training for a teacher's job at HBO	May-Jun 2007
Orientation & training for a teacher's job at HBO	May-Jun 2008
► **Journal club**	
Literature discussion "Phytopathology"	2005 - 2008
► **Individual research training**	
Subtotal In-Depth Studies	*9.9 credits**

4) Personal development	*date*
► **Skill training courses (highly recommended)**	
Basiscursus Didaktiek	02-03-04 Apr, 24-25-26 Jun 2008
Cursus Collegegeven	08-15-22 May 2008
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	32,3

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study*